# Enhancing Activity of the Anaphase Promoting Complex to Reverse Multiple Drug Resistant Behaviour in Breast Cancer

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By:
Cordell VanGenderen

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## Abstract

The development of multiple drug resistant (MDR) behaviour in cancer poses a significant challenge to effective treatment, yet this is not a rare event and occurs in up to 20% of breast, lung, colon, and hematological malignancies. MDR is defined as the presence of clinical resistance to cancer therapy including resistance to medical therapies not previously used. MDR can be innate, or acquired over the course of treatment. The underlying causes of MDR are clearly multifactorial, and there are several common themes that promote the behaviour: genomic instability, impaired stress response pathways, and dysregulated mitosis all promote MDR behavior. These cellular mechanisms exist as an interrelated network of pathways, as disruption of one often impacts another, such as the loss of mitotic regulation promoting genomic instability. This accumulation of mutations may then impair the activity of various stress responses pathways, thereby reducing the apoptotic response to chemotherapy and promoting MDR behaviour. The Anaphase Promoting Complex (APC) is an evolutionarily conserved, multi-subunit E3 ubiquitin ligase enzyme that targets selected proteins for proteasomal degradation during mitosis and G1. Its functions include activation of stress response pathways and regulated progression of mitosis, which protects the cell from genomic instability. Previously, a correlation had been established between dysfunction of the APC and cancer progression, primarily indicated by the accumulation of many APC substrates noted in multiple cancers. Recently, this relationship was directly implicated in the progression of cancer into an MDR state in canine lymphomas where relapse correlated with impaired APC function and remission restored APC activity. Therefore, we hypothesize that enhancing APC activity will subsequently restore chemosensitivity in MDR cancer.

In yeast, novel peptides were identified that bound and activated the *Saccharomyces cerevisiae* (*S. cerevisiae*) APC. When stably expressed in the innately MDR breast cancer cell line MDA-MB-231, two of these peptides significantly enhanced APC activity. Significantly, peptide expression restored cytotoxic sensitivity to doxorubicin in this MDR cell population in accordance with relative APC activity, with greater APC activation producing greater chemosensitivity. An investigation of FOXO3A-dependent stress response pathways (suggested by previous studies) demonstrating a functional interaction between APC activity and yeast FOXO orthologs, to induce stress responses. We revealed FOXO3A signaling activity was increased upon APC-activating peptide expression, an elevation in FOXO3A activity, and therefore predicted a concurrent increase in stress-related apoptosis. As anticipated, peptide expression increased apoptosis with or without chemotherapy exposure. Peptide-dependent APC activity unexpectedly augmented mitotic dysregulation observed as an increased number of mitotic catastrophes, including chromosomal mis-segregation and micronuclei formation. The consequences of mitotic catastrophes are elevated genomic instability and DNA damage, which were found to be elevated. While this behaviour is typically considered to be tumorigenic, this phenomenon may enhance the cytotoxic effect of chemotherapy by inducing cell death via priming the cells with elevated genomic damage. Our results indicate that activation of the APC serves to enhance chemosensitivity in MDR cancer; however, the complicated mechanisms related to APC activity warrants further investigation into its relationship with MDR behaviour.

## Acknowledgements

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Lastly, I would like to thank the University of Saskatchewan College of Medicine and the Canadian Institutes of Health Research for funding both myself and my research.

## Dedication

I dedicate this thesis to my parents, Herman and Lillian for their perpetual support.

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## List of Abbreviations:

| | |
|---|---|
| 231 | MDA-MB-231 |
| ABC | ATP-Binding Cassette |
| APC | Anaphase Promoting Complex |
| Apcin | APC Inhibitor |
| BAD | Bcl2-Associated Agonist of Cell Death |
| Bcl | B-cell Lymphoma |
| BCRP | Breast cancer resistance protein (ABCG2) |
| BER | Base Excision Repair |
| BRCA | Breast Cancer Susceptibility Gene |
| BSA | Bovine Serum Albumin |
| BUB3 | Budding Uninhibited by Benzimidazole 3 |
| BUBR1 | Budding Uninhibited by Benzimidazole-Related 1 |
| CDC20 | Cell Division Cycle 20 |
| CDH1 | Fizzy and Cell Division Cycle 20 Related 1 |
| CDK | Cyclin Dependent Kinase |
| cPARP | Cleaved PARP |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl Sulfoxide |
| DOX | Doxorubicin |
| DDR | DNA Damage Response |
| DTT | Dithiothreitol |
| DUSP1 | Dual Specificity Protein Phosphatase 1 |
| ECL | Enhanced Chemiluminescent Reagent |
| EDTA | Ethylenediaminetetraacetic Acid |
| EGFR | Epidermal Growth Factor Receptor |
| EGTA | Egtazic acid |
| Em | Emission wavelength |
| EMI1 | Early Mitotic Inhibitor 1 |
| ER | Estrogen Receptor |
| EtOH | Ethanol |
| Ex | Excitation wavelength |
| FBS | Fetal Bovine Serum |
| Fkh | Forkhead Box Transcription Factor |
| FOXO | Forkhead Box O |
| GEF | Guanine Exchange Factor |
| GSK3α | Glycogen Synthase Kinase 3 Alpha |
| HA | Haemagglutinin |

| | |
|---|---|
| HER2 | Human Epidermal Growth Factor Receptor 2 |
| HRP | Horseradish Peroxidase Conjugates |
| HSP90B1 | Heat Shock Protein 90 Beta Family Member 1 |
| IGF | Insulin/Insulin-Like Growth Factor IGF |
| IKK | Inhibitor of Nuclear Factor kappa-B kinase |
| INFAR1 | Interferon Alpha/Beta Receptor 1 |
| IRS | Insulin Receptor |
| IPTG | Isopropyl β- d-1-thiogalactopyranoside |
| JAK | Janus Kinase |
| LB | Luria Broth |
| LBA | Luria Broth Ampicillin |
| M2I-1 | MAD 2 Inhibitor 1 |
| MAD1 | Mitotic Arrest Deficient 1 |
| MAD2 | Mitotic Arrest Deficient 2 |
| MAPK | Mitogen-activated protein kinase |
| MCC | Mitotic Checkpoint Complex |
| MCM | Mini-chromosome Maintenance Protein |
| MDR | Multiple Drug Resistance |
| MDR-1 | Multidrug Resistance Protein 1 (ABCB1) |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| mTOR | Mammalian target of rapamycin |
| MTORC | mTOR complex |
| NEK2A | NIMA-Related Kinase 2 |
| NFκB | Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells |
| NFKBIA | NFκB Inhibitor Alpha |
| NLS | Nuclear Localization Signal |
| PARP | Poly(ADP) Ribose Polymerase |
| PBS | Phosphate Buffered Saline |
| PBST | PBS Tween 20 |
| PCR | Polymerase Chain Reaction |
| PDK1 | 3-Phosphoinositide-Dependent Protein Kinase-1 |
| PGDF | Platelet Derived Growth Factor |
| PH | Pleckstrin-homology |
| PHLPP | PH Domain and Leucine rich repeat Protein Phosphatases |
| PI3K | Phosphoinositide-3-kinase |
| PIP2 | Phosphatidylinositol 4,5-bisphosphate |
| PIP3 | Phosphatidylinositol (3,4,5)-trisphosphate |
| PKC | Protein Kinase C |
| PKRD1 | Protein Kinase D1 |
| PLK1 | Polo-Like Kinase 1 |

| | |
|---|---|
| PR | Progesterone Receptor |
| Pro-TAME | pro-Tosyl-L-Arginine Methyl Ester |
| PPP1 | Protein Phosphatase 1 |
| PPP2 | Protein Phosphatase 2 |
| PTEN | Phosphatase and Tensin Homolog |
| RAPTOR | Regulatory-Associated Protein of TOR 1 |
| RIPA | Radioimmunoprecipitation |
| RPM | Revolutions per Minute |
| RPSKCA3 | Ribosomal S6 kinase alpha-3 |
| RPSKCB1 | Ribosomal S6 kinase beta-1 |
| RT | Room Temperature |
| RTK | Receptor Tyrosine Kinase |
| SAC | Spindle Assembly Checkpoint |
| *S. cerevisiae* | *Saccharomyces cerevisae* |
| SDS | Sodium Dodecyl Sulfate |
| SHC1 | SHC transforming protein 1 |
| SOS1 | Son of Sevenless 1 |
| SOCS3 | Suppressor of Cytokine Signaling 3 |
| STAT | Signal Transducer and Activator of Transcription |
| TE | Tris-EDTA Buffer |
| TGFβ | Transforming Growth Factor beta |
| TGFBR1 | TGFβ Receptor 1 |
| TNBC | Triple Negative Breast Cancer |
| TTK | TTK protein kinase |
| TTKi | TTK inhibitor |
| TrxA | Thioredoxin |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

## Organization of Thesis

This thesis is based on three manuscripts. As two of the manuscripts are unpublished, the decision for the format of this thesis was to take a hybrid approach, incorporating both elements of the traditional thesis format, as well as separate chapters 4 and 5 representing each of the two unpublished manuscripts. The first published manuscript is a review article titled "*The role of Anaphase Promoting Complex activation, inhibition and substrates in cancer development and progression*", which was accepted for publication by the journal "*Aging"* on July 12, 2020. The manuscript is a review of the identified roles of the Anaphase Promoting Complex and its substrates in the development and progression of cancer, and serves as the basis for Chapter 1, Introduction. I am the first author and the co-authors are Drs Troy Harkness and Terra Arnason.

The second manuscript is in preparation and has a working title of "*Activation of the yeast Anaphase Promoting Complex using peptides that bind Apc10 increases resistance to stress and lifespan, and has anti-cancer potential*" and is a combination of yeast and cancer work involving the peptides. Results presented in the thesis from this manuscript are solely my contribution. This paper has the co-first authors Rachel Harris, myself, and Dr. Gerald Davies. This manuscript serves as the basis for Chapter 4.

The third manuscript is also in preparation and focuses solely on utilizing the peptides to understand the role of APC dysfunction in MDR cancer, and will be co-first authored by myself and MSc candidate Gabrielle Mercier. This manuscript serves as the basis for Chapter 5, and the results presented in this thesis are limited to the work I have performed. As both of these manuscripts are unpublished, the data referencing to, but not presented, will be referred to as "data unpublished".

Lastly, the reference "Arnason, under review" refers to the manuscript "*Activation of the Anaphase Promoting Complex reverses multiple resistant cancer"* and is currently under review for publication in *Scientific Reports*. This paper served as both the foundation and the inspiration for many of the investigations performed in this thesis. The manuscript reports on the discovery that the development of MDR behaviour in canine lymphomas corresponds with the development of APC dysfunction, restoration of chemosensitivity was associated with normalized APC activity.

# Chapter 1. Introduction

## 1.1 General Characteristics of Multiple Drug Resistant Behaviour

Cellular defects incurred during cancer development and progression alter the specific behaviours of individual malignancies. One commonly developed behaviour is multiple drug resistance (MDR), a phenomenon whereby the resistance to one treatment or chemotherapy modality results in resistance to multiple unrelated therapies that have not been previously used (Holohan, *et al.,* 2013; Housman *et al.,* 2014). Treatment resistance is a frequent occurrence, particularly in malignancies of the breast, prostate, hematological system and gastrointestinal system (Komarova 2006). A diverse array of mechanisms are known to contribute to MDR behaviour including impairment of stress response and apoptotic pathways (Fernald and Kurokawa, 2013; Miyashita and Reed, 1993), the expression of drug efflux pumps (Choi, 2005; Austin Doyle *et al.,* 1998), genomic instability (Lee *et al.,* 2011; Sansregret *et al.,* 2018), and the restoration of defective DNA repair mechanisms (Bonano *et al.,* 2014; Edwards *et al,* 2008; Husain *et al.,* 1998). The underlying mechanisms of MDR are complex regardless of the cancer type, and can include some or many of these cellular changes. These pathways may be defective early on during cancer development, or acquired later after initial responses to treatment, and MDR behaviour is often described as being innate or acquired (Holohan, *et al.,* 2013; Housman *et al*, 2014). Given that each pathway itself experiences a unique series of defects to promote its aberrant behaviour, effective treatment of MDR behaviour poses a significant challenge (Gerlinger *et al.,* 2012; Vogelstein *et al.,* 2013). Despite this, there are several commonly accepted biomarkers of MDR behaviour. Perhaps the most notable markers of MDR behaviour are members of the ATP-binding cassette (ABC) transporter family which act as non-specific drug efflux pumps, the overexpression of which prevents the accumulation of sufficient therapeutic agent in the cell to induce its affects (Choi, 2005). Currently, detection of MDR responses to first line therapy result in a switch to rescue therapies with higher risk profiles, or in fact a change to palliative comfort therapy only, as there is no current effective treatment for MDR malignancy available. Emerging novel approaches for MDR therapy include considerations for either a combinatorial approach, or a synthetic lethality approach where inhibition of mechanisms complimenting the cancer behaviour induce cell death (Crystal *et al.,* 2014; Liu and Tewari, 2016).

## 1.2 Models of MDR Behaviour in Breast Cancer

Breast cancer is the most common malignancy to affect women in the world, accounting for 25% of all female cancers (Ghoncheh *et al.,* 2016). Breast cancer is known to have high rates of treatment resistance even 20 years after initial successful responses to therapy, with rates that differ depending on the hormone receptors expressed on the cancer cells (Kramer *et al.,* 2019; Reddy *et al.,* 2018). Histological grading of breast cancer has identified several subtypes of breast

cancer. Categorizing the subtypes of breast cancer is based in part on the presence of three different receptors: estrogen (ER), progesterone (PR) and human epidermal growth factor receptor 2 (HER2) (Foulkes *et al.,* 2010). Breast cancers expressing either ER and/or PR are often treated with endocrine therapies to block proliferation signals arising through these receptors (including the treatments tamoxifen and toremifene) (Printz, 2014). Breast cancers whose development are driven by the overexpression of HER2 are referred to as HER2+, and similar to ER and PR positive cancers, are treated with therapies targeting the HER2 receptor (including trastuzumab and pertuzumab) (Oh and Bang, 2020). In contrast are those breast cancers that do not express any of the classic receptors and therefore cannot benefit from blockade in the same way and are referred to as triple negative breast cancer (TNBC). TNBC typically exhibits more aggressive characteristics than ER, PR, or HER2+ cancers, and TNBC patients have the lowest 3-year survival rate of the three histological grades and requires a reliance on traditional chemotherapies as receptor-targeted therapies are ineffective (Kramer *et al.,* 2019). Furthermore, TNBC breast cancer patients experience elevated instances of resistance to chemotherapy and metastasis, resulting in patients who have shorter relapse-free periods and lower survival rates (D'Amato *et al.,* 2015; Liedtke *et al.,* 2008; Tan *et al.,* 2008). Of diagnosed breast cancers, 10-17% are TNBC leaving few effective therapeutic options for a large population of patients (Tan *et al.,* 2008).

### 1.3 General Characteristics of the Anaphase Promoting Complex

The anaphase promoting complex (APC) is an evolutionarily conserved multi-subunit E3 ubiquitin ligase (E3 Ub-ligase) enzyme named after its first recognized function, that of promoting the segregation of chromosomes during mitosis, thus initiating anaphase. However, the role of the APC in cellular behaviours has expanded greatly beyond this initial function, and the APC is now recognized to participate in interphase regulation (Choudhury *et al.,* 2016; Wei *et al.,* 2004), cell cycle arrest (Basserman *et al.,* 2008; Sudo *et al.,* 2001), and induction of stress responses (Malo *et al.,* 2016; Postnikoff *et al.,* 2012; Simpson-Lavy *et al.,* 2009). When impaired, the APC is known to result in genomic instability (Engelbert *et al.,* 2008; Greil *et al.,* 2016; Kim *et al.,* 2011), cell cycle dysregulation (Greil *et al.,* 2016; Park *et al.,* 2008; Sigi *et al.,* 2009; Sudo *et al.,* 2001) and diminished stress responses (Postnikoff *et al.,* 2012; Simpson-Lavy *et al.,* 2009; Sudo *et al.,* 2001), all key aspects of cancer development and progression (Hanahan and Weinberg, 2011; Harkness, 2018). The mammalian APC is highly related structurally and functionally to that of lower eukaryotes including the yeast (*S. cerevisiae*). In humans, the APC is composed of 14 subunits, with key subunits including APC2 and APC11 which are critical for the E3-ligase catalytic activity (Thornton *et al.,* 2006). The CDC27 subunit, which binds to CDC20 (cell division cycle 20) and CDH1 (CDC20 and Fizzy homolog 1) coactivator proteins at mutually exclusive positions in the cell cycle (Vodermaier *et al.,* 2003). The APC10 subunit contributes to E3 ligase activity via substrate recruitment to the inner cavity of the APC structure (Buschhorn *et al.,* 2011).

The APC is principally regulated through the exclusive binding of one of two coactivators, CDC20 and CDH1, forming $APC^{CDC20}$ and $APC^{CDH1}$ respectively. At the initiation of mitosis, the

APC is inhibited through impaired association to either coactivator, then becomes active upon CDC20 binding (**Figure 1.1**). During mitotic exit CDC20 dissociates from the APC, which is then bound by CDH1 for the remainder of mitosis until CDH1 degradation in G1 (Sivakumar and Gorbsky, 2015). The availability for CDH1 and CDC20 to bind the APC is regulated through acetylation of both CDC20 and CDH1 and their deacetylation is a key regulatory event impacting APC activity, as acetylation prevents their respective bindings to the APC (Kim *et al.,* 2011).

The primary recognition motif of proteins selected and targeted by the APC for degradation is the destruction box (D-box, RxxLxxI/VxN), which exists on a multitude of APC substrates and is recognized by both the $APC^{CDC20}$ and $APC^{CDH1}$ complexes (Glotzer *et al.,* 1991; Pfleger and Kirschner, 2000). Both coactivators contain a WD40 protein-binding domain that interacts with the APC substrates (Kraft *et al.,* 2005), and assists with APC and E2-ubiquitin interactions to promote APC E3 activity (Brown *et al.,* 2014; Brown *et al.,* 2015; Van Voorhis and Morgan, 2014). A variety of secondary motifs also exist that are recognized by either $APC^{CDH1}$ or $APC^{CDC20}$ exclusively including the KEN box (KENxxD) (Pfleger *et al.,* 2000) and L box (LXEXXXN) (Sullivan and Morgan, 2007), which are targeted by $APC^{CDH1}$, and an LR motif which is targeted by $APC^{CDC20}$ (Sedgwick *et al.,* 2019). These secondary motifs act to target specific proteins rather than as general Ub-dependent degradation motifs.

### 1.4 Normal APC Function during Mitosis

As shown in Figure 1.1, during metaphase, the Spindle Assembly Checkpoint (SAC) is active, delaying mitotic progression until all sister chromatids are securely attached to the mitotic spindle (Sivakumar *et al.,* 2015). The SAC is maintained by the mitotic checkpoint complex (MCC), a multi-subunit complex that inhibits APC activity until all kinetochores are properly secured to a microtubule (Monda and Cheeseman, 2018). The MCC subunit component mitotic arrest deficient 2 (MAD2), when associated with the kinetochore via the MAD1 subunit, binds to the N-terminus of CDC20, which then associates with budding uninhibited by benzimidazole 3 (BUB3) and BUB3-related 1 (BUBR1) to form the tetrameric MCC. The MCC complex binds two CDC20 molecules, suggesting that MCC also interacts with CDC20 bound to APC. In the cryo-EM structure, MCC-CDC20 binds to $APC^{CDC20}$, where MCC-CDC20 occupies the large $APC^{CDC20}$ central cavity (Izawa and Pines, 2015; Yamaguchi *et al.,* 2016). BUBR1 interacts with both CDC20 molecules, thereby disrupting the ability of both CDC20 molecules to bind substrate. This occurs because BUBR1 encodes the APC recognition motifs, D-box and KEN-box, through which CDC20 binds (Di Fiore *et al.,* 2016). Once microtubules are properly attached to the kinetochores of chromosomes, the SAC becomes inactivated and releases CDC20 so it can in turn activate the APC by forming $APC^{CDC20}$ (**Figure 1.1**, Kramer *et al.,* 2000).

The inactivation of the SAC begins the first phase of APC activity whereby formation of the $APC^{CDC20}$ complex promotes anaphase by the ubiquitination (and subsequent proteasomal degradation) of multiple protein targets. Two prominent proteins involved in chromosomal segregation are Securin (which is targeted by the APC for degradation) and Separase (which is not

directly targeted by the APC). Securin is an inhibitory chaperone of Separase, which acts by allosterically altering the conformation of bound Separase to prevent binding to target proteins (Luo and Tong, 2017). Separase is a cysteine protease that cleaves the kleisin subunit of cohesin. Cohesin acts to bind sister chromatids together and cleavage of the kleisin subunit results in dissolution of the cohesin ring binding sister chromosomes together, and induces chromosomal segregation (Lin *et al.,* 2016; Gligoris *et al.,* 2014). The APC drives anaphase by polyubiquitinating Securin, targeting it for degradation, and enabling Separase activity. The newly activated Separase then triggers chromosomal segregation by cleaving the kleisin subunit. While bound to CDC20 the APC will also self regulate in a feedback loop where it targets multiple SAC components to prevent further APC inhibition (Sitray-Shevan *et al.,* 2018; Song and Rape, 2010). The APC will also target Cyclin B1 for degradation. Cyclin B1 functions by binding and activating cyclin dependent kinase 1 (CDK1) which phosphorylates multiple targets to promote mitosis, including several APC subunits and the second APC activator, CDH1 (Kraft *et al.,* 2003; Kramer *et al.,* 2000). The phosphorylation of APC subunits promotes $APC^{CDC20}$ activity, while inhibiting interaction of CDH1 with the APC. Thus, the degradation of Cyclin B1 results in the loss of phosphorylation of CDH1, permitting its binding to the APC forming $APC^{CDH1}$ (Kraft *et al.,* 2003; Kramer *et al.,* 2000).

The formation of $APC^{CDH1}$ initiates the targeting of a new suite of protein degradation targets and the next phase of APC activity that permits a regulated mitotic exit. These targets include the proteins, CDC20, FOXM1, residual Cyclin B1, and multiple mitotic kinases including NIMA related 2 (NEK2A), and Aurora A/B, the degradation of which is necessary to maintain a regulated cell cycle (Zhang *et al.,* 2016). The binding of CDH1 at this stage to the APC also triggers a negative-feedback loop where the APC simultaneously targets the CDH1 protein for degradation as the cells progress through G1 (Kramer *et al.,* 2000; Visintin *et al.,* 1997). Residual CDH1 will still bind the APC and the $APC^{CDH1}$ complex will continue to function throughout G1 (Castro *et al.,* 2005). The role of the APC in regulated mitotic progression is essential for the maintenance of chromosomal integrity, genomic stability, and cell cycle regulation (Greil *et al.,* 2016; Sudo *et al.,* 2001).

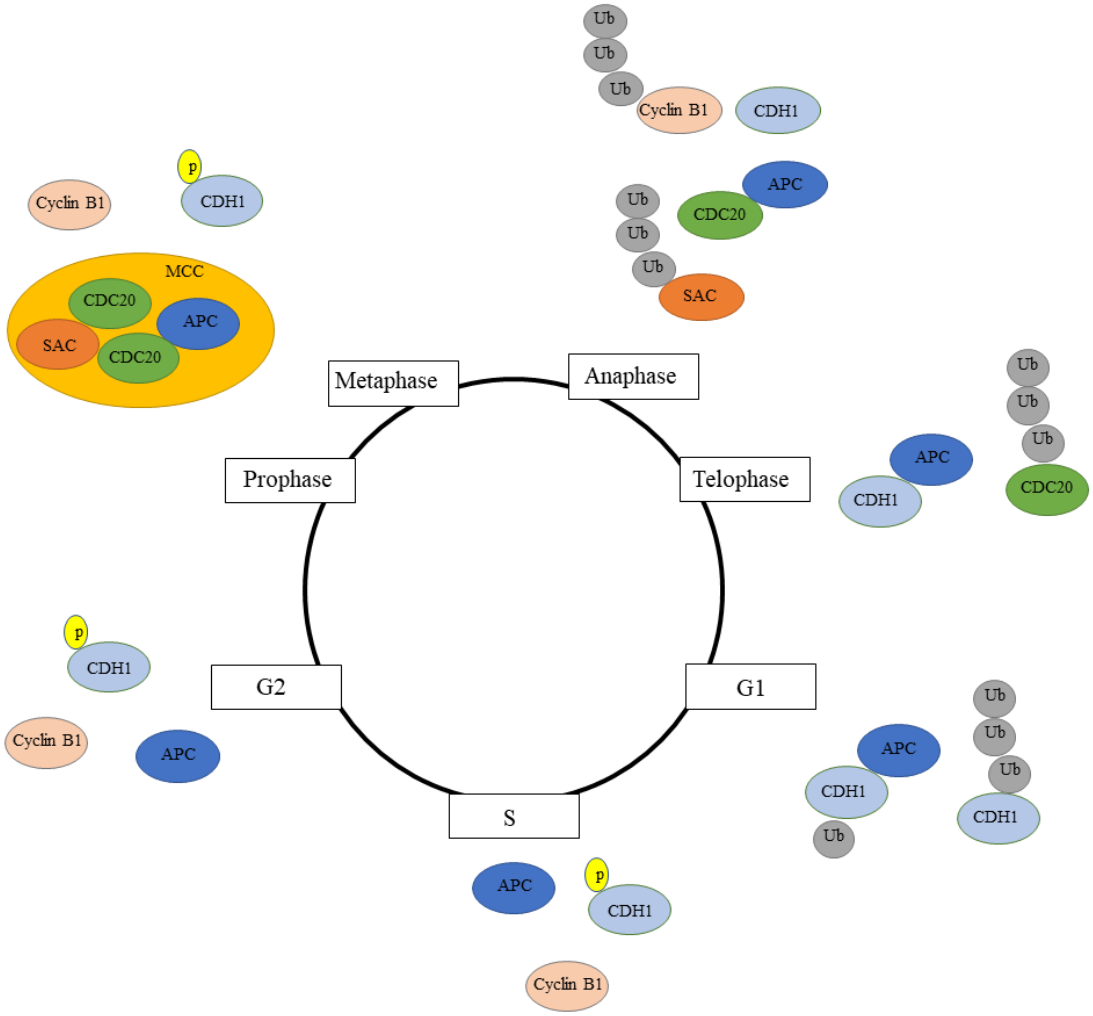



**Figure 1.1 Regulation of the APC throughout the cell cycle.** Beginning in metaphase, the APC is inhibited through SAC binding CDC20, and CDH1 inhibitory phosphorylation. Upon chromosomal alignment, the SAC becomes inactive and CDC20 binds APC, initiating the polyubiquitination of the SAC and Cyclin B1. The loss of Cyclin B1 permits CDH1 replacing CDC20 during mitotic exit, inducing CDC20 polyubiquitination. The APC remains bound to CDH1 during G1, however acting in a negative-feedback loop whereby it degrades CDH1. Residual CDH1 is phosphorylated by Cyclin B1. The APC remains inactive from S phase until chromosomal alignment in metaphase.

## 1.5 APC Regulation by the Mutually Exclusive Coactivators, CDC20 and CDH1

### 1.5.1 CDC20 and APC regulation

CDC20 has been identified as being an oncogene, and both overexpression and augmented protein abundance have been correlated with poor prognosis for several unrelated cancer types including brain astrocytoma (Ding *et al.,* 2017), gastric (Ding *et al.,* 2014), breast (Karra *et al.*,2014), colorectal (Wu *et al.,* 2013), prostate (Mao *et al.,* 2016), and pancreatic cancers (Chang *et al.,* 2012). Increased CDC20 abundance drives a dysregulated mitotic cycle by overwhelming the inhibitory capacity of the SAC, forcibly activating the APC despite an active SAC, and inducing mitotic slippage (the improper progression of mitosis despite continual SAC activity) (Bonaiuti *et al,* 2018; Pan and Chen, 2004). Overexpression of *CDC20* is one mechanism that could lead to accumulation of CDC20 protein (Bonaiuti *et al,* 2018), as would its failure to be degraded efficiently by the proteasome in a Ub-dependent manner. One obvious mechanism would be the dysfunction of the APC E3-ligase activity itself through altered post-transcriptional modifications or impaired subunit expression, resulting in inefficient CDC20 degradation. It is thought that by driving improper APC activity (and therefore mitosis) that CDC20 primarily contributes to tumorigenesis. However, the observation that overexpression of *CDC20* is accompanied by the overexpression of a host of other genes associated with APC impairment in other cancers (Zhang *et al,* 2019), including overexpression of other APC substrates, indicates that it may be APC impairment, not specifically CDC20 overexpression, that is important for cancer development, in at least some cases.

### 1.5.2 CDH1 and APC regulation

The potential role that CDH1 plays in cancer development and progression is more complicated than CDC20, as it has been demonstrated to possess both tumor suppressor and oncogenic functions (Ishizawa *et al,* 2017). The generation of heterozygous $CDH1^{+/-}$ mice are haploinsufficient and incur elevated rates of cancer formation indicating an overall tumor suppressive function (Garcí-Higuera *et al.,* 2008; Ishizawa *et al,* 2017). Loss of CDH1 activity is also connected with increased rates of chromosomal abnormalities (Engelbert *et al.,* 2008; Garcí-Higuera *et al.,* 2008; Greil *et al.,* 2016), elevated sensitivity to DNA damage (de Boer *et al.,* 2016; Ishizawa *et al,* 2011; Ishizawa *et al,* 2017), insufficient loading of Mini-Chromosome Maintenance proteins (MCMs) (Greil *et al.,* 2016), and premature S phase entry (Choudhury *et al.,* 2016; Greil *et al.,* 2016; Sigi *et al.,* 2009; Wei *et al.,* 2004). These abnormalities result from the loss of key regulatory functions of CDH1, which includes cell cycle arrest upon nutrient and genotoxic stress (defined as stressors on the cell that induce genetic damage) (Basserman *et al.,* 2008; Gao *et al.,* 2009, Petersen *et al.,* 2000; Simpson-Lavy *et al.,* 2009; Sudo *et al.,* 2001), regulation of S phase entrance (Choudhury *et al.,* 2016; Park *et al.,* 2008; Wei *et al.,* 2004), and promoting mitotic exit (Hatano *et al.,* 2016; Wäsch *et al.,* 2002).

The complicated relationship between CDH1 and cancer behavior has been experimentally elucidated by CDH1 silencing in a B-cell acute leukemia model. Initially, CDH1 impairment

promoted DNA damage induced-apoptosis, however long-term inhibition resulted in the development of resistance to radiation-based therapy (Ishizawa *et al.,* 2017). Furthermore, in B-cell acute leukemia patients, CDH1 deficiency predicted a prolonged remission period, and elevated life expectancy (Ishizawa *et al.,* 2017). The CDH1 protein has also been found to be elevated in malignant as compared to normal tissue controls (Lehman *et al.,* 2007). The oncogenic behaviour of CDH1 is primarily a result of its antagonism to the SAC and can act to induce mitotic slippage (Nagai and Ushimaru, 2014; Park *et al.,* 2018; Toda *et al.,* 2012). $APC^{CDH1}$ overactivity from either *CDH1* overexpression, or loss of the $APC^{CDH1}$ inhibitor, early mitotic inhibitor 1 (EMI1), may also result in DNA re-replication through the over-degradation of Geminin (Sørensen *et al.,* 2000; Yuichi *et al.,* 2007). In G2 and S phase, Geminin acts to inhibit CDT1, which is responsible for initiating DNA replication. Therefore, inappropriate loss of CDT1 inhibition may result in DNA re-replication and induce gene duplication and aneuploidy (Machida and Dutta, 2007; Sørensen *et al.,* 2000; Wohlschlegel *et al.,* 2000). The wide variety of CDH1-associated activities demonstrates its complicated role in cancer progression, and warrants further investigation. Through the cellular behaviours, the overaccumulation of CDH1 may also contribute to MDR behaviour through generation of genomic instability and mitotic slippage.

## 1.6 Role of Genomic Instability in Cancer Development and MDR Behaviour

### 1.6.1 DNA damage response pathways

Genomic instability refers to the accrual of genomic damage incurred through chromosomal instability and nucleotide instability (Giam and Rancati 2015; Sansregret *et al.,* 2018; Tubbs, 2017). Chromosomal instability involves the partial or entire addition and deletion of chromosomes, while nucleotide instability refers to mutations of individual nucleotide residues and DNA breaks (Giam and Rancati 2015). One common factor promoting genomic instability is the impairment of the DNA damage response (DDR) pathways. Germline mutations of DNA repair genes including *BCRA1, BRCA2, MLH1*, and *MSH2* are established to predispose transformation into a variety of cancer types, as the cells experience a reduced capacity to repair the normal stochastic accrual of DNA damage (Bouwmen and Jonkers, 2012; Leach *et al.,* 1993; Moynahan *et al.,* 1999; Papadopoulos *et al.,* 1994; Wooster *et al.,* 1995). DDR pathways may also be impaired through the inhibition of DNA damage sensors, such as the key regulatory proteins, p53 (Ohashi *et al.,* 2015), ataxia-telangiectasia mutated (ATM, Guo *et al.,* 2002), ataxia telangiectasia and Rad3 related (ATR, Göhler *et al.,* 2011), Chk1/2 (Sørensen *et al.,* 2005; Sun *et al.,* 2014). Malignancies are commonly found to contain these loss-of-function mutations, or otherwise experience impaired activity/expression of these sensing proteins (Yazinski and Zhu, 2016). DDR pathway defects prevent the recognition of DNA damage and the normal initiation of either repair or apoptosis (Göhler *et al.,* 2011; Guo *et al.,* 2002; Ohashi *et al.,* 2015; Yazinski and Zhu, 2016), increasing the likelihood of acquiring *de novo* mutations in surviving malignant cells that trigger further cancer development and progression. The impairment of one DDR pathway results in oncogene addiction, where the proper functioning of a secondary pathway is essential for survival in the cell. This

phenomenon is exemplified when homologous repair pathways are inhibited through loss-of function *BRCA2* mutations, preventing the repair of double strand breaks (Mateo *et al.,* 2015). This induces reliance on repair single strand break DDR pathways to substitute the loss of double-strand repair normally performed by homologous repair pathways. Pharmacological inhibition of base excision repair (BER) pathways, which are crucial for single-strand break repair, in *BRCA2* defective cells through poly(ADP–ribose) polymerase (PAPR) inhibitors results in reduced cellular viability and cell death (Ashworth, 2008).

In contrast to cellular mechanisms designed to detect DNA damage, cancer behavior can also change due to the altered activity of various DNA repair mechanisms, and specifically contribute to MDR behaviour. This occurs through an increase in DNA repair reducing the accumulation of DNA damage. This prevents the accrual of lethal DNA damage through chemo- and radio-therapy, resulting in MDR behaviour (Edwards *et al,* 2008; Husain *et al.,* 1998; Kwon *et al.,* 2007; Kirschner and Melton, 2010; Sakai *et al.,* 2008). This phenomenon has been observed through the elevated expression of *ERCC1* (excision repair cross complementing), a key component of nucleotide excision, recombination, double strand break, and interstrand crosslink repair pathways, inhibits the apoptotic response to chemotherapy and promotes drug resistance (Kwon *et al.,* 2007; Kirschner and Melton, 2010). A similar phenomenon has been documented with *BRCA1* overexpression inhibiting the response to cisplatin. However, the effect on chemosensitivity is dependent on the chemotherapy class used, as *BRCA1* overexpression simultaneously promotes the sensitivity to taxanes (Husain *et al.,* 1998). Lastly, another alteration causing increased DDR activity may also arise through gain of function mutations. As noted in *BRCA2,* an intragenic deletion restores defective *BRCA2* activity and promotes resistance to cisplatin (Sakai *et al.,* 2008; Edwards *et al,* 2008).

The role of DDR pathways in promoting MDR behaviour has led to investigations of DDR inhibitors to restore chemosensitivity and promote cellular apoptosis. Often cancer cells will become reliant on the activity of one specific DDR pathway, leading to targeting DDR pathways through a synthetic lethal approach. As mentioned previously, *BRCA 1/2*-impaired cancers typically rely on PARP activity to compensate for the impaired homologous repair pathways within the cell, yet treatment of these *BRCA*-impaired cancers with PARP inhibitors has demonstrated effective anti-tumorigenic effects (Ashworth, 2008; Farmer *et al.,* 2005, Mateo *et al.,* 2015). Furthermore, utilizing chemotherapy cocktails and therapies, such as pairing a chemotherapeutic with a DDR inhibitor, has been demonstrated to provoke sufficient DNA damage to efficiently trigger cell death (Bonano *et al,* 2014; Crystal *et al.,* 2014; Selvakumaran *et al,* 2003).

### 1.6.2 Chromosomal instability and aneuploidy

Chromosomal instability refers to errors relating to chromosome structure, number, and fragmentation (Giam and Rancati, 2015; Sansregret *et al.,* 2018). Multiple mechanisms contribute to chromosomal instability including dysregulated/error prone mitosis and replication stresses (Burrell *et al.,* 2013; Comailis *et al.,* 2016; Crasta *et al.,* 2012; Ohashi *et al.,* 2015). Mitotic errors

may result in improper chromosomal segregation of a portion or entire chromosomes, generating aneuploidy and creating severe chromosomal damage in the daughter cells (Comailis *et al.,* 2016; Crasta *et al.,* 2012; Ohashi *et al.,* 2015). The relationship between chromosomal instability and mitosis will be further discussed in section 1.7. Polyploidy similarly arises from an error in mitosis, occurring when the cell fails to undergo cytokinesis, and instead directly enters interphase from prophase or metaphase (Ohashi *et al.,* 2015). The presence of chromosomal instability provides a significant source of the heterogeneity and genomic instability within the genomes of the cancer cell population, through induction of gene amplification, or haploinsufficiency of key oncogenes and tumor suppressors, thus driving tumor progression (Gerlinger *et al.,* 2012; Lee *et al,* 2011).

In a healthy cell, the genotoxic and proteotoxic (stresses incurred in the cell resulting in aberrant protein regulation) stresses induced by chromosomal instability would either induce apoptosis, or otherwise trigger cell death. To avoid this cellular death, cancer cells frequently develop mutations to compensate for the severe genotoxic and proteotoxic stresses induced by genomic instability. Key mutations identified in yeast are the loss of function mutations to the de-ubiquitinases, Ubp3 or Ubp6, indicating promotion of proteasomal degradation acts to protect cancer cells from proteotoxic stresses and promotes cell survival (Dodgson *et al.,* 2016; Torres *et al.,* 2010). There does, however, remain a point where chromosomal instability passes a tolerable level and the cell will experience death (Birkbak *et al.,* 2011). Frequently this occurs during mitotic catastrophes. Currently there is not a universally accepted definition of mitotic catastrophe, and there is debate as to whether cell death necessarily be included, especially in the case of malignant cells. Therefore, we will define mitotic catastrophes as errors in mitosis that would induce death in a healthy cell. Aside from errors during mitosis (lagging chromosomes, failure of chromosomes to align along a metaphase plate etc.) characteristic cellular morphologies of mitotic catastrophes post-mitosis are micronuclei (extranuclear chromosomes), multi-nuclei formation, and asymmetric nuclei formation (Caruso *et al.,* 2011).

### 1.6.3 Adaptational advantage of genomic instability in malignancy

Each cancer cell in the population experiences a unique combination of chromosomal and genetic defects, producing a heterogenous genome of the cell population (Gerlinger *et al.,* 2012). These defects may include loss of function mutations in key tumor suppressor genes, or overexpression of oncogenes through gene amplification induced by aneuploidy. It is through accumulations of a unique series of defects within individual cancer cells that genomic instability provides an adaptational advantage driving numerous cancer behaviours including MDR, metastasis, apoptosis suppression, and heightened cell proliferation (Chen *et al.,* 2012 Lee *et al,* 2011; Yates *et al.,* 2017). This heterogeneity in particular poses a challenge for targeted therapies whose anti-tumorigenic effect occurs from focusing on specific proteins that may be up or downregulated on average within a population, while select cells may be protected (Sotillo *et al.,* 2010).

## 1.7 The Promotion of MDR Behaviour through Dysregulated Mitosis

### 1.7.1 Mechanisms promoting dysregulated mitosis

One of the most common causes of a dysregulated mitosis is through the process of mitotic slippage. Mitotic slippage refers to processes by which chromosomal segregation occurs despite mitotic arrest appropriately induced by sustained SAC activity (Wäsch, 2011). Mitotic slippage is achieved through the premature activation of APC despite sustained active SAC repression. This can be accomplished through the overexpression of *CDC20*, as excess protein abundance prevents the SAC from inhibiting APC activity due to an inability to sufficiently sequester the excess CDC20 protein. This allows the free CDC20 to form $APC^{CDC20}$ and promote anaphase, with mitotic slippage occurring as a result (Bonaiuti *et al,* 2018; Pan and Chen, 2004). A second mechanism whereby the APC may overcome SAC inhibition is through premature CDH1 activity. As the SAC does not directly inhibit CDH1 activity, failure of the regulatory mechanisms that inhibit APC activation via CDH1 results in mitotic slippage, as $APC^{CDH1}$ can prematurely target Securin for degradation (Nagai *et al.,* 2014; Park *et al.,* 2018; Toda *et al.,* 2012). This occurs principally if Cyclin B1 activity is impeded, as $CDK1^{Cyclin\ B1}$ phosphorylation of CDH1 prohibits CDH1 from binding the APC. This dysfunction may occur if there is insufficient Cyclin B1 expressed during mitosis, or if there is a deficiency of ATP which is necessary for CDK1 to perform its inhibitory phosphorylation of CDH1 (Park *et al.,* 2018). Together, then, lowering the CDC20 and Cdh1 abundance should mitigate mitotic slippage. This could be accomplished by enhancing Ub-mediated degradation of these APC subunits through APC activation.

Mitotic dysregulation may also arise from the aberrant overexpression of mitotic kinases, including NEK2A, polo-like kinase 1 (PLK1) and Aurora A/B, which has been linked with cancer development and progression (Pérez de Castro *et al.,* 2007). This is due to their roles in regulation of key aspects of mitosis including, mitotic arrest, spindle assembly, and chromosome separation. For instance, NEK2A promotes centrosome maturation and chromosome condensation. However, in cancer cells, the overexpression of NEK2A dysregulates mitosis through inducing centrosome amplification, the generation of mitotic spindle defects, and premature centriole splitting. These defects may result in multi-nucleation and chromosomal mis-segregation during mitosis (Xia *et al.,* 2015). Overactive NEK2A may also activate the SAC component MAD2 to induce mitotic arrest. This arrest is not always beneficial for cellular health however, and can induce aneuploidy if performed aberrantly (Liu *et al.,* 2010). These mitotic kinases are also APC degradation substrates, again highlighting the possibility of therapeutic APC-activation in having a benefit on mitotic fidelity by normalizing the amount of these mitotic kinases.

### 1.7.2. Mechanism through which dysregulated mitosis promotes MDR behaviour

While mitotic dysregulation may occur through either mitotic slippage or aberrant kinase activity, the consequences are similar; the presence of mitotic dysregulation impacts multiple aspects of cancer behaviour. The most prominent consequence of mitotic dysregulation is the dysregulated proliferation of cells, a characteristic behaviour of malignancies (Riffell *et al.,* 2009).

The prolonged mitotic arrest prior to cells experiencing mitotic slippage induces DNA damage, and the resolution of mitotic arrest through slippage instead of apoptosis carries this elevated DNA and chromosomal damage into the daughter cells (Dalton *et al.,* 2007; Quignon *et al.,* 2007). Mitotic dysregulation may induce chromosomal mis-segregation which may result in aneuploidy or micronuclei formation (Crasta *et al.,* 2012, Ohashi *et al;* 2015, Zhu *et al.,* 2014). Mitotic slippage also produces an innate resistance to microtubule poisons. This is due to microtubule poisons preventing the proper formation of mitotic spindles, therefore deriving their therapeutic effects through inducing a prolonged mitotic arrest to trigger apoptosis. However, the circumvention of SAC activity prevents this outcome, therefore microtubule poisons are unable to induce apoptosis (Anand *et al.,* 2003; Jiang *et al.,* 2003; Sudo *et al.,* 2004; Zhu *et al.,* 2014).

### 1.7.3. *In vitro* and *in vivo* APC targets as possible cancer therapies

Due to the importance of mitosis in cancer development and progression, multiple stages of mitosis have been carefully investigated for therapeutic potential (Huang *et al.,* 2009; Liu *et al.,* 2019). Many early chemotherapy treatments were microtubule poisons and function as described above. Microtubule poisons are divided into two categories, microtubule stabilizers (taxol and docetaxel) and microtubule stabilizers (vinblastine and vindesine) and are a staple treatment across a variety of cancer forms (Dominguez-Braur *et al.,* 2015) Cancers often develop resistance to these microtubule poisons (Jiang 2011, Huang 2009). This has led to the development of direct APC inhibitors (mimicking SAC activity without requiring its presence) to induce mitotic arrest and directly prevent slippage. Pharmacological agents include APC inhibitor (Apcin) and pro-Tosyl-L-Arginine Methyl Ester (pro-TAME) both of which inhibit the CDC20-APC interaction, preventing the formation of $APC^{CDC20}$ and thereby inhibiting APC activity (Sackton *et al.,* 2014; Zeng *et al.,* 2010). Pro-TAME also inhibits the second APC coactivator, CDH1, from binding the APC and effectively eliminates APC activity (Zeng *et al.,* 2010). Both agents have demonstrated antitumorigenic results *in vitro* (Gao *et al.,* 2018; Sackton *et al.,* 2014), although due to the essential functions of the APC in healthy cells, *in vivo* toxicity is a concern (Zhang *et al.,* 2014).

APC activators have also been commercially developed. Opposing mitotic arrest as a therapeutic route, therapies have also been developed that induce mitotic slippage through inhibiting SAC activity. Pharmacological agents have been developed to target the TTK and MAD2 subunits of the SAC, referred to as TTK inhibitors (TTKi, Wang *et al.,* 2019) and MAD2 inhibitor 1 (M2I-1, Kastl *et al.,* 2015) respectively. TTKi and M2I-1 treatments derive their therapeutic effect from preventing the sequestration of CDC20 by the SAC (thereby increasing the availability of CDC20 for APC binding and activation), driving a dysregulated mitosis. This, in effect, induces mitotic slippage to promote catastrophic chromosomal mis-segregation and genomic instability, inducing cell death through mitotic catastrophes (Kastl *et al.,* 2015; Li *et al.,* 2019; Maia *et al.,* 2018; Thu *et al.,* 2018; Wengner *et al.,* 2016). *In vitro* M2I-1 and TTKi have demonstrated anti-tumorigenic effects both individually and when complemented with microtubule treatments, with several TTKi treatments currently under clinical trials *in vivo* (Maia *et al.,* 2018; Thu *et al.,* 2018; Wengner *et al.,* 2016). Targeted therapies for mitotic kinases

frequently found to be overexpressed in cancer including NEK2A, PLK1, and Aurora A/B have been developed, with some agents moving into clinical trials (Gutteridge *et al.,* 2016; Liu *et al.,* 2019; Tsuda *et al.,* 2017; Xia *et al.,* 2015).

### 1.8 Stress Response and Apoptotic Pathways

#### 1.8.1 Suppression of apoptosis to promote MDR behaviour

An essential aspect in cancer cell biology is the avoidance of cell death mechanisms (Hanahan and Weinberg, 2011). To achieve this, malignancies often directly suppress apoptotic pathways through altered regulation of apoptosis signalling genes including the B-cell lymphoma (Bcl) family of proteins (Adams and Cory, 2007) and the Caspases (Olsson and Zhivotovsky, 2011). This protects the cancer cell from cell death despite the presence of severe cellular defects that would typically result in apoptosis. Impairing apoptosis to permit cell survival also prevents chemotherapy from effectively inducing apoptosis, impeding its therapeutic efficacy, and promoting MDR behaviour. The restoration of apoptotic pathways reverses this phenomenon, and restores chemosensitivity (Adams and Cory, 2007; Friedrich *et al.,* 2001; Miyashita and Reed, 1993).

#### 1.8.2 Regulation of apoptotic pathways through stress response networks

Cancer may also inhibit apoptosis indirectly through the suppression of stress response pathways, which normally play a critical role in the powerful promotion of apoptotic processes (Gardai *et al.,* 2004; Kinoshita *et al.,* 1997; Thayyullathil *et al.,* 2011). The activity of stress response pathways is intrinsically linked to proliferative pathways, with the promotion of stress responses impeding proliferative pathways and vice versa (Landau *et al.,* 2012). In cancer, proliferative pathways often experience enhanced activity promoted by growth factor signaling, via prominent signaling pathways including insulin/insulin-like growth factor (IGF) and epidermal growth factor (EGF), often silencing stress responses (Pros *et al.,* 2013). Cancer cells also alter intracellular signaling to promote growth. These signalling pathways respond to direct energy and nutrient sensing within the cell performed primarily (but not exclusively) by 5’ AMP-activated kinase (AMPK) and mammalian target of rapamycin (mTOR) complex 1 (MTORC 1) (DeBerardinis *et al.,* 2008; Tokunaga *et al.,* 2004). Impairment of stress response pathways, particularly through activity of the PI3K signaling pathways has been demonstrated to promote MDR behaviour (Goler-Baron *et al.,* 2012; Miller *et al.,* 2011; Tokunaga *et al.,* 2006; Zhou *et al.,* 2013).

#### 1.8.3 PI3K signaling pathway and stress responses

Phosphoinositide 3-kinases (PI3K) signaling is regulated through growth factor signaling including IGF, EGF, and platelet derived growth factor (PGDF) signaling. Receptor activation upon binding of their respective ligands induces autophosphorylation of PI3K proteins to promote their activity (Singh *et al.,* 2016). A primary function of PI3Ks is the phosphorylation of

phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to form phosphatidylinositol (3,4,5)-trisphosphate ($PIP_3$). $PIP_3$ induces the localization of proteins containing a pleckstrin-homology (PH) domain to the cell membrane where they become active through a complex series of interactions (Bartholomeusz *et al.,* 2012). Prominent proteins directly activated through PI3K signaling are mTORC2 (regulatorily and functionally distinct from mTORC1, but share mTOR as the kinase domain), AKT, and 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Bartholomeusz *et al.,* 2012). Proteins activated by PI3K signaling then proceed to promote proliferation and impair stress responses and apoptosis. This pathway is directly impaired by the tumor suppressor, PTEN (Phosphatase and tensin homolog) which dephosphorylates $PIP_3$ into $PIP_2$, preventing protein localization to the membrane and silencing PI3K signaling (Roy *et al.,* 2010). Sustained PI3K signalling is a key promoter of proliferation in cancer (Foukas *et al.,* 2010; Zhao *et al.,* 2006).

### 1.8.4 AKT signaling and stress responses

After localization to the membrane, AKT receives activating phosphorylation from PDK1 and mTORC2 (Sarbassov *et al.,* 2005). AKT acts as a regulator for numerous pathways including nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), apoptosis, and Forkhead Box O (FOXO) (Brunet *et al.,* 1999; Datta *et al.,* 1997; Jablonski *et al.,* 2015). The net affect of AKT activity is the suppression of apoptosis, and promotion of the cell cycle (Datta *et al.,* 1997; Xiao *et al.,* 2010). The FOXO protein family is a group of transcription factors that promote stress responses by transcribing proteins to inhibit cell cycle progressing and induce apoptosis. Proteins transcribed by the FOXO family include Death Receptor 4/5, $p27^{Kip1}$, Bim, and Bcl-6 (Greer and Brunet, 2005; Roy *et al.,* 2010; Shoeb *et al.,* 2013). AKT1 is inhibited by protein phosphatase 1 and 2 (PPP1, PPP2), and PH domain and Leucine rich repeat Protein Phosphatases (PHLPPs) which dephosphorylates the activating phosphorylating events to impair AKT activity (Xiao *et al.,* 2010). Besides inducing MDR behaviour through the suppression of apoptotic mechanisms, AKT interacts with the ABC transporter family, promoting the transport vesicles containing BCRP (an ABC family member) to the cell membrane (Goler-Baron *et al.,* 2012), and is correlated with elevated abundance of other members of the ABC family including multidrug resistance protein 1 (MDR1), and multidrug resistance-associated protein 1 (MRP1) (Zhou *et al.,* 2013). The activity of AKT promotes MDR behaviour (Miller *et al.,* 2011; Tokunga *et al.,* 2006), and inhibition of AKT in cancer has been demonstrated to induce apoptosis and enhance sensitivity to chemotherapy, with many of the observed affects a result of upregulated FOXO3A activity (Cleary *et al.,* 2010; Roy *et al.,* 2010; Shoeb *et al.,* 2013).

### 1.8.5 Cell cycle checkpoints and stress responses

Cell cycle regulation is dependent on the proper functioning of cell cycle checkpoints to arrest cell cycle progression to address proteotoxic, nutrient, and genomic stresses. Cancer progression induces heavy stressors on the cell, and therefore cancer cells often experience downregulation of cell cycle checkpoints to permit (inappropriate) cell cycle progression (An *et al.,* 2015; Zhang *et al.,* 2014). Adequate checkpoint activity is necessary for the initiation of

multiple DNA repair mechanisms, and loss of checkpoint activity significantly increases the cellular stress as the burden of damage accumulates (Göhler *et al.,* 2011; Sørensen *et al.,* 2005). Commonly, loss-of-function mutations in key cell cycle checkpoint proteins prevent the cell cycle arrest, and not unexpectedly are also involved in cancer development (Malumbres and Barbacid 2009; Tutt and Ashworth 2002). Cell cycle checkpoints can be overridden through the overexpression of antagonistic proteins, as demonstrated by the overexpression of Aurora A that suppresses the normal expression of *p53, BRCA 1/2*, *ATR, Chk 1* and *RAD51*, thereby indirectly preventing cell cycle arrest (Sun *et al.,* 2014). Similarly, the overexpression of microRNAs also inhibit the expression of crucial checkpoint proteins (An *et al.,* 2015, Zhang *et al.,* 2014).

As cancer progresses into an MDR state, reactivation of cell checkpoints may contribute to MDR behaviour. An indicative phenotype of this behaviour is a reduction in the proliferative rate of cancer cells, as restored checkpoints slows cell cycle progression. A reduced proliferative rate has demonstrated an ability to protect cancer cells from both chemo- and radio-therapy as well as intrinsic cell death mechanisms (Harrington *et al.,* 1994; Giancotti 2013). This reduced proliferative rate is often a result of checkpoint re-activation as it reduces the load of genotoxic and proteotoxic stresses in the cell likely by permitting partial genome repair and avoidance of cellular death, thereby promoting MDR behaviour (Ferrao *et al.,* 2012; Morgan *et al.,* 2010). This role of checkpoints in initiating DNA repair has resulted in them being targeted for inhibition as an anti-cancer approach. Pharmacological inhibition of Chk1 has demonstrated efficacy, both *in vitro* and *in vivo,* in preventing homologous repair pathways in cancer cells and enhancing the cytotoxic response to radiotherapy through impairment of activation of the G2 checkpoint (Ferrao *et al.,* 2012; Morgan *et al.,* 2010).

### 1.8.6 Normal APC function and induction of cell cycle arrest

The $APC^{CDH1}$ complex can initiate cell cycle arrest at multiple stages of the cell cycle. At the G2/M transition, $APC^{CDH1}$ acts in conjunction with CDC14B and PLK1 to prevent progression into mitosis in the event of DNA damage. During the G2/M transition arrest, the phosphatase CDC14B will translocate to the nucleoplasm from the nucleolus and activate $APC^{CDH1}$ via dephosphorylation of the CDH1-inhibitory phosphorylation events blocking its recruitment to the APC (Basserman et al. 2008). $APC^{CDH1}$ will then target PLK1 for degradation preventing the phosphorylation of Claspin (which initiates DNA repair pathways) by PLK1. This phosphorylation event results in protein instability, therefore $APC^{CDH1}$ activity serves to protect Claspin and promote DNA repair (Bassermann *et al.,* 2008, Sudo *et al.,* 2001).

The APC may also induce cell cycle arrest in response to nutrient stresses (Sudo *et al.,* 2001; Simpson-Lavy *et al.,* 2009; Wirth *et al.,* 2004). This interaction has been studied utilizing rapamycin, a pharmacological inhibitor of the nutrient signalling kinase, mTOR. Therefore, rapamycin serves as a pharmacological mimetic to nutrient stresses. When tested in *in vitro* $CDH1^{-/-}$ chicken cell lines (DT40), rapamycin is unable to induce G1 cell cycle arrest (Sudo *et al.,* 2001). This is a result of altered CDK2 and retinoblastoma (Rb) pathway signaling. Upon rapamycin treatment, wild type cells lose Rb phosphorylation, allowing the induction of G1 arrest, but in

CDH1$^{-/-}$ cells, Rb phosphorylation is maintained with continued cell cycle progression (Sudo *et al.,* 2001). In *S. cerevisiae* Cdh1 has been demonstrated to protect the cell from ethanol, caffeine, and hyperosmotic stress, as yeast cells lacking *CDH1* still progress through the cell cycle, but are sensitive to multiple stresses (Simpson-Lavy *et al.,* 2009). The stress sensitivity appears to be due to elevated stability of Clb2 (ortholog to human Cyclin B2) and Hsl1 (ortholog of human NIM1-related Kinase) from a partially impaired APC E3-ligase activity that fails to fully degrade these targets, and which in turn continues to drive cells through the G2/M transition despite the incurred cellular damage. Inhibition of the APC in quiescent cells drives their return to the cell cycle (Cappell *et al.,* 2016; Wirth *et al.,* 2004). This indicates that APC activity is required both for entrance to, and maintenance of, cell cycle arrest.

Studies in *S. cerevisiae* have revealed the complicated networks that the deacetylation enzyme Sir2 (the yeast orthologue of SIRT2) influences. Sir2 is an important stress response protein in *S. cerevisiae*, and it is tightly connected with a stress response network that interacts with the Forkhead Box transcription factors (Fkh), Fkh1 and Fkh2 (orthologues to mammalian FOXO proteins) (Linke *et al.,* 2013). In *S. cerevisiae*, under stress conditions, the APC and Fkhs work together to induce a response to stress (Malo *et al.,* 2016; Postnikoff *et al.,* 2012). Furthermore, when stress is encountered, Sir2 is recruited to Clb2 promoters in a Fkh1-dependent manner to repress Clb2 expression and stall the cell cycle (Linke *et al.,* 2013).

### 1.9 Investigating the role of the APC in cancer progression and MDR behaviour

Previous studies have indicated that the APC becomes dysfunctional as cancer develops and this correlates with more aggressive tumor behaviours (Arnason unpublished; Lehman *et al.,* 2007; Wang *et al.,* 2003; Wild al*.,* 2018). During the progression from benign into malignant tumors it is common to observe an elevated abundance of APC substrates including the coactivators CDH1 and CDC20. This also coincides with an elevated abundance of the APC inhibitor early mitotic inhibitor 1 (EMI1), indicating APC activity is likely impaired during cancer development, as the APC is incapable of sufficiently degrading its target proteins. The impairment of APC activity has also been observed in canine lymphoma as the malignancy progresses from chemoresponsive tumors into a treatment resistant/MDR malignancy *in vivo* (Arnason, unpublished). Therefore, impaired APC activity correlates with cancer progression, particularly into MDR behaviour; however, a direct causal relationship has yet to be established between APC dysfunction and cancer progression. Therefore, in Chapter 4 we will modulate the activity of the APC to examine the impact of APC activity on MDR behaviour and restoring chemosensitivity.

Current methods of altering APC activity are based on either promoting (TTKi, M2I-1) or inhibiting (Apcin, pro-TAME) coactivator binding. From these inhibitor studies we understand the effects of manipulating APC activation and inhibition to achieve therapeutic effects; however, a knowledge gap remains regarding the impact of APC-regulatory elements outside of directly modulating the CDC20- and CDH1-coactivator binding. There is a broad array of regulatory mechanisms influencing APC function based upon regulating complex stability (Tran *et al.*, 2010), and post-transcriptional modifications (Zhang *et al.*, 2016) that affect APC E3 ligase activity. The

impacts on cellular behaviour through modulating APC behaviour by these mechanisms has not been thoroughly investigated, and potentially differ from alterations to CDH1 or CDC20 binding. This aspect will be investigated in Chapter 4, where novel peptides which have demonstrated a capability for binding and augmenting APC activity will be endogenously expressed in an innately MDR breast cancer to examine the effects of altered APC activity on MDR behaviour.

There is also the question of the phenotypic alterations imparted upon cancer cells by impaired APC activity, and how augmenting APC activity may affect these phenotypes. There are multiple cellular pathways that are expected to be altered by augmented APC activity that warrant investigation. The relationship between stress responses and APC activity have been explored in eukaryotic model systems. Of particular note are the observed interactions between the APC and Fkh proteins in yeast to promote stress responses (Malo *et al.,* 2016; Postnikoff *et al.,* 2012,). This specific pathway has not yet been examined in mammalian system. In mammalian systems, FOXO activity is regulated through AKT signaling, therefore the AKT/FOXO network will be examined for alterations upon enhanced APC activity. This pathway is also particularly important as elevated AKT activity is a known promoter of MDR behaviour (Goler-Baron *et al.,* 2012; Miller *et al.,* 2011; Zhou *et al.,* 2013). We will also investigate the implications of augmented APC activity with regards to mitotic regulation. Given the essential aspect of APC activity in mitotic progression, altering APC activity is likely to affect mitotic regulation in cancer cells, in a manner that may support or impair cancer growth and progression. Both of these aspects of cellular behaviour, stress response signaling, and mitotic regulation will be addressed in Chapter 5.

## Chapter 2. Hypothesis and Objectives

**Primary Hypothesis:**

Decreased APC activity is associated with more aggressive cancer behaviour and correlates with the presence of a MDR response to chemotherapy. Therefore, we hypothesize that restoration of APC activity will subsequently restore chemosensitivity.

**Secondary Hypothesis:**

We hypothesize that through known APC interactions with stress response pathway proteins and influence on mitotic regulation, the targeted enhancement of APC activity will correct these defects that promote MDR behaviour, and thereby allow for reversal of chemoresistance.

**Objective 1: Generating an in vitro model of APC activity in MDR cancer**

Currently no established methods exist of directly acting on the APC to enhance its activity. Recently a yeast 2-hybrid library was generated which identified novel peptides capable of binding discrete APC subunits. Several of these peptides have subsequently demonstrated an ability in *S. cerevisiae* models of APC defects to reverse incurred phenotypes, indicating peptide expression enhances APC activity. Due to evolutionary conservation of the APC, these peptides may act in a similar manner on the human APC to enhance its activity. To utilize these peptides in human models, they will be subcloned into mammalian constitutive expression vectors. Once cloned these peptide expressing vectors will be stably transfected into an innately MDR breast cancer cell line to endogenously express the peptides. This will permit us to study the phenotypes that arise in MDR cancer when APC activity is enhanced.

**Objective 2: Establish a relationship between APC activity and MDR behaviour**

APC E3 ubiquitin ligase activity will be indirectly measured through examining the relative abundances of select substrates to identify the peptides most effective at enhancing APC activity. A decrease in the accumulation, as identified by Western blotting of APC substrates, will indicate an enhancement of the APC E3 ligase activity. The peptides identified as being the most effective at enhancing APC activity, as indicated by the greatest reduction substrate levels across multiple targets, will be selected for chemosensitivity assays. These assays will consist of treatment with the cytotoxic agent doxorubicin (DOX) followed by a reporter assay to quantitate the viability of the cell population following treatment. This will be the most direct test of our hypothesis.

**Objective 3: Investigate the mechanisms relating APC activity to MDR behaviour**

Multiple potential mechanisms exist that link enhanced APC activity with MDR behaviour. Two mechanisms which are likely relevant to the APC reversing MDR behaviour are the induction of stress response pathways and the promotion of a regulated mitosis; two processes which are often disrupted in MDR cancers. The specific stress pathway that will be examined is the AKT-FOXO3A pathway, which when manipulated to inhibit AKT promotes FOXO3A activity

and initiates a stress response and apoptosis. The second mechanism being investigated is the role of the APC in promoting a regulated mitotic cycle. Enhanced activity of the APC may act to correct the dysregulated mitosis normally occurring in MDR cells. To measure this, fluorescent microscopy utilizing a DNA stain will permit visualization of mitotic events. Quantification of improper mitotic events (referred to as mitotic catastrophes) will be the measure of how mitotic regulation is after peptide expression. One consequence of a dysregulated cell cycle is the accumulation of DNA damage, which will also be analyzed through γH2A.X abundance, which is recruited to double strand DNA breaks. This will serve as the test of our secondary hypothesis.

# Chapter 3. Methods and Materials

## 3.1 Yeast Peptide Cloning into a Human Expression Vector

Polymerase chain reaction (PCR) amplification was performed on each peptide using the original yeast plasmids as a template. The orientation of the peptides from the N-terminus: SV40 nuclear localization signal (NLS, sequence PKKKRKV), B42 transcriptional activating domain, Haemagglutinin (HA, sequence YPYDVPDYA) tag, thioredoxin (TrxA), peptide, TrxA. All PCR reactions utilized the same 3' primer, binding after the C-terminus of the second TrxA sequence. Two 5' primers were utilized in separate reactions to produce two distinct insert sequences from the same original sequence. One 5' primer bound 3' of the B42 sequence (producing a DNA fragment without the NLS, or B42 sequences) and one primer bound 5' of the NLS (producing a DNA fragment with the NLS and B42 sequences). All amplified sequences contain HA-TrxA-peptide-TrxA. PCR sequences were then incubated with 0.5 units/μL Taq polymerase (New England Biolabs, Whitby, ON, Canada) at 70°C for 30 min in 1x Taq buffer (supplemented with 0.2 mM dNTP and 2.5 mM $MgCl_2$) to produce thymidine overhangs. PCR products were then purified with Zymo DNA purification and concentration columns following manufacturers protocol (Burlington, ON, Canada). After purification, insert sequences were ligated into pGEM-T Easy vector (Promega, Madison WI, USA) via TA ligation following the manufacturer's instructions and transformed into the *Escherichia coli* (*E. coli*) strain DH5α (Thermo Fisher Canada, Mississauga, ON, Canada). Transformed *E. coli* were then incubated on Luria Broth (LB)-Agar plates (0.5% w/v yeast extract, 1% w/v tryptone, 171 mM NaCl, 0.2% agar) supplemented with the antibiotic ampicillin (0.1 mg/mL) and blue/white screening (20 mg/mL X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside] and 100 mM IPTG [Isopropyl β- d-1-thiogalactopyranoside]). White bacterial colonies were then cultured in LB (with 0.1 mg/mL ampicillin, LBA) for plasmid amplification at 37°C for 16 hrs with agitation. The plasmids were then purified using phenol-chloroform purification (Appendix A.4). Insert sequences were then verified by restriction digests using BamHI (Thermo Fisher) and XhoI (Thermo Fisher) following the manufacturer's protocol. Insert fragments were then purified via gel extraction (Appendix A.5) and ligated into pcDNA 3.1 (Thermo Fisher) previously restricted with BamHI and XhoI using T4 ligase following manufactures protocol. Plasmids were then transformed into DH5α and plasmid DNA purified using the phenol-chloroform extraction method. Sanger sequencing performed by the National Research Council (Saskatoon, SK, Canada) confirmed proper insertion of the peptides into pcDNA3.1 and confirmed the correct reading frames between epitope tags and proteins.

## 3.2 Cell Culture and Plasmid Transfection

The TNBC cell line MDA-MB-231 (231) was commercially obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in Dulbecco's Modified Eagle's Media (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine

serum (FBS, Invitrogen), 1X Ab/Am (100U/mL penicillin, 100 mg/mL streptomycin, 0.25 µg/mL of Amphotericin B [Invitrogen]) unless otherwise stated. Incubators were maintained at 37˚C and 5% $CO_2$. Cells were transfected using Lipofectamine 3000 (Thermo Fisher), following the manufacturer's protocol. Stable integration of the plasmids was performed by persistent exposure to the neomycin analogue geneticin (Invitrogen) in 2%, 3%, and 4% increments for 48 hr each.

### 3.3 Western Blotting

Western blotting was performed as previously described (Davies *et al.,* 2009). Cells were harvested from the cell culture dishes in 1 mL 1x phosphate buffered saline (PBS; 0.067M $PO_4$) with a rubber policeman. Cells were then pelleted at 10,000 rpm for 2 minutes and the PBS decanted. The pellet was then resuspended in 100 µL 1X radioimmunoprecipitation (RIPA) buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% glycerol, 0.5 mM Ethylenediaminetetraacetic Acid [EDTA], 0.1 mM Egtazic acid [EGTA], 0.1% sodium dodecyl sulfate [SDS] plus 1X mammalian cell anti-protease cocktail [MilliporeSigma Canada, Oakville, ON, Canada]). Cells were then lysed via pulse sonication and centrifuged at 18,000 rpm and 4˚C for 5 minutes. The supernatant was decanted, and the pellet discarded. The commercial Bradford assay (BioRad Protein Reagent; BioRad Laboratories, Hercules, CA, USA) quantified protein content in the lysate based on a standard curve in a SmartSpec 3000 (BioRad). A lysate aliquot was then diluted into 100µL of RIPA (to reach desired concentration) then protein sample of protein was denatured with a 5x sample buffer (5x loading buffer [250 mM Tris HCl pH 6.8, 10% w/v SDS], 15% v/v 2-mercaptoethanol, 30% v/v glycerol, and trace bromophenol blue) and boiled for 2 min. Lysates were then run an acrylamide gel between 7.5% and 15% at 150V. The gel was then transferred onto a nitrocellulose membrane (BioRad) in 1X transfer buffer (0.19 M glycine, 25 mM tris base, 10% v/v methanol) at 90V for 1.5 hrs. After transfer, membranes were stained with Ponceau S (0.1% (w/v) Ponceau S [Thermo Fisher] in 1% v/v acetic acid) as a non-specific protein stain and imaged with a photocopier to document the relative loads. Ponceau S was then washed from the membrane with two washes in 1x PBS with 1% Tween 20 (PBST) for 5 min each, with agitation. The membrane was then blocked with a 5% w/v skim milk 1X PBST solution at room temp (RT) for one hr. Primary antibody incubation occurred with either 1:1000 (total protein) or 1:500 (phosphor-protein) dilutions in 5% skim milk PBST overnight at 4˚C. Membranes were then washed 3 times for 10 min in 5% skim milk PBST at RT. The membrane was then incubated in a 1:10,000 secondary antibody-horseradish peroxidase conjugates (HRP) dilution in 5% skim milk PBST solution for 1 hr at RT. Three 10 min washes were then performed with 1X PBST. This was followed by a 5 min incubation with enhanced chemiluminescent reagent (ECL, BioRad) permitted imaging with a VersaDoc and analysis with the QuantitiyOne software (BioRad; version 4.6.9).

### 3.4 Cellular Viability Assays

Viability assays were performed as previously described (Davies *et al.,* 2009). Cells were grown to 50% confluence and subject to doxorubicin hydrochloride (DOX; Pfizer, Brandon,

Manitoba, Canada) treatment for 48 hours in DMEM. The cells were then incubated for 2 hours with phenol red free DMEM media supplemented with 10% FBS, and 2% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; MilliporeSigma) solution. Cells were then solubilized with 99.5% dimethyl sulfoxide (DMSO) for absorbance spectrophotometry at λ570 nm with a SmartSpec 3000 (BioRad).

### 3.5 Immunofluorescence

Cells were grown on coverslips to 60% confluence. They were fixed by incubation in 4% paraformaldehyde for 10 min at RT then washed twice with 1X PBS and stored at 4°C. Cells were permeabilized by incubation in 0.5% Triton X-100 for 10 min followed by 0.05% Triton X-100 for 5 min. Cells were incubated with a 1:200 primary antibody dilution in 1% bovine serum albumin (BSA) for 1 hour in a humidity chamber at RT. Cells were washed with 0.05% Triton X-100 then incubated with a 1:200 secondary fluorescent antibody dilution in 1% BSA for 1 hour in a humidity chamber at RT. Cells were then washed with 0.05% Triton X-100 for 5 min at RT. Cells were then placed on cover slips onto 4′,6-diamidino-2-phenylindole (DAPI) droplets and the edges sealed with nail polish and stored at 4°C.

### 3.6 Kinome Array

The array was performed by collaborators in Dr. Nappers' lab (University of Saskatchewan), whereas the data analysis and pathway construction was done by us. The array was performed as described previously (Määttanen *et al.*, 2013). Briefly, cells from our strains of interest were harvested from cell culture dishes into 1x PBS with a rubber policeman and pelleted at 10,000 rpm for 2 minutes, and decanted. The pellet was then resuspended in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, 1 mM NaF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM PMSF, 1 µg/ml pepstatin A, and 2 mM dithiothreitol [DTT] [all products from Sigma-Aldrich unless otherwise indicated]) and vortexed. The homogenate was diluted 8-fold in ice cold lysis buffer, vortexed, and incubated for 20 min on ice to permit lysis. The lysates were then further diluted to achieve a protein concentration between 1.0-1.5 mg/mL as according to the Bio-Rad Bradford method. A 70 µl aliquot of supernatant was mixed with 10 µL of activation mix ((50% glycerol, 500 µM ATP [New England BioLabs], 60 mM MgCl2, 0.05% vol/vol Brij 35, 0.25 mg/ml BSA) and incubated on the array for 2 h at 37°C. Arrays were then washed with 1% Triton X-100.

Slides were then submerged in a phosphor-specific fluorescent ProQ Diamond phosphoprotein stain (Invitrogen) while being agitated for 1 hour followed by to 3 washes in a 20% acetonitrile (EMD Biosciences [VWR distributor], Mississauga, Ontario, Canada), 50 mM sodium acetate (MilliporeSigma) pH 4.0 destain solution for 10 min. A final wash with $ddH_2O$ was performed prior to 20 min air drying. Slides were then centrifuged at 300 x *g* for 2 min to remove residual moisture. Arrays were analyzed with a GenePiX Professional 4200A microarray scanner (MDS Analytical Technologies, Toronto, Ontario, Canada) at 530 to 560 nm with a 580-

nm filter. Images were collected using the GENEPix 6.0 software (MDS). Spot intensity signals were collected as the mean of pixel intensity using the local feature background intensity calculation with the default scanner saturation level. The local background intensities were subtracted from foreground intensities, and the resulting value transformed with variance-stabilizing normalization to eliminate variance-versus-mean dependence and normalize the data for easier analysis. The resulting data set contained signal intensities from 3 technical repeats (per phosphorylation site per peptide).

### 3.7 Reagents

**Antibodies:**
pan-AKT ab8805 (Abcam; Cambridge, MA, USA)
p-AKT S473 ab81283 (Abcam)
BCRP sc-377176 (Santa-Cruz Biotechnology; Dallas, TX, USA)
Cyclin A2 ab38 (Abcam)
Cyclin B1 C8831 (SigmaMilliPore)
CDC20 PA5-34775 (Thermo)
FOXO3A ab70315 (Abcam)
p-FOXO3A ab47285 (Abcam)
γ-H2A.X H5912 (SigmaMillipore)
HURP ab80684 (Abcam)
MDR1 sc55510 (Santa-Cruz)
NEK2A ab115731 (Abcam)
PPP1CA ab137512 (Abcam)
p-PPP1CA T320 ab62334 (Abcam)
Rabbit HRP 170-6515 (BioRad)
Mouse HRP 170-6516 (BioRad)

**Enzymes:**
Taq Polymerase M0267S (New England Biolabs)
Phusion HS II F549S (Thermo)
BamHI FD0054 (Thermo Fisher)
XhoI FD0694 (Thermo Fisher)
T4 Ligase M0202S (New England Biolabs)

**Kits:**
Caspase Kit ab219915 (Abcam)
PGEM-T Easy A1360 (Promega)
Lipofectamine 3000 L3000008 (Thermo Fisher)
E. coli (DH5α) 18265017 (Thermo Fisher)

# Chapter 4. Modulating Cancer Behavior by Enhancing APC Activity in Multiple Drug-Resistant Breast Cancer

## 4.1 Introduction

### 4.1.1 Overview of the Anaphase Promoting Complex

The Anaphase Promoting Complex (APC) is an E3 ubiquitin (Ub) ligase and performs a myriad of functions necessary for the proper functioning and health of the cell. As a fundamental function, E3 ubiquitin ligases select and target specific proteins for regulated degradation by the proteasome. For the APC, this target selection occurs in coordination with the cell cycle (Sivakumar *et al.,* 2015). There are multiple critical consequences of normal APC action including promoting a regulated mitotic progression, inducing stress response pathways and therefore the balance between apoptosis and cellular progression, as well as ensuring genomic stability through regulating mitosis, DNA replication and DNA repair mechanisms. Therefore, disruption of APC activity results in loss of cell cycle regulation, genomic instability, and impaired stress responses (Basserman *et al.,* 2008; Greil *et al.,* 2016; Harkness, 2018; Simpson-Lavy *et al.,* 2009; Sodu *et al.,* 2001). These phenotypes are common characteristics of cancer development and progression into more aggressive behaviours, and therefore loss of APC activity may promote malignant behaviors (Hanahan and Weinberg, 2011).

The APC is highly controlled both through regulated suppression and activation mechanisms, including common themes of post translational modifications in concert with the position of the cell cycle, as well as the subunit composition of the APC complex itself. The APC is activated by the mutually exclusive physical association of two coactivators, CDC20 and CDH1, each with unique roles in APC activity and at distinct positions within the cell cycle (Kramer *et al.*, 2000; Visitin *et al.*, 1997). In addition, the protein(s) selected for Ub-mediated degradation can depend on which of the two coactivators is bound (Pfelger *et al.*, 2000). An example of regulated APC suppression is elegantly presented by considering the sequestration of CDC20 by the SAC prior to metaphase (Pan and Chen, 2004). During metaphase (prior to chromosomal alignment) the APC remains inactive as the SAC sequesters CDC20 and CDH1 is at low levels and unable to bind and activate the E3. Upon proper alignment of the chromosomes, the SAC becomes inactivated and releases CDC20, which then binds to the APC to form $APC^{CDC20}$. The now active $APC^{CDC20}$ is then free to perform polyubiquitination, to promote chromosomal segregation, and progression through anaphase, and telophase by targeting proteins for degradation that normally prevent progression (Luo and Tong, 2017; Sivakumar *et al.*, 2015). During mitotic exit CDC20 will dissociate from the APC, being replaced by CDH1 forming $APC^{CDH1}$ (Kramer *et al.,* 2000; Visintin *et al.,* 1997). This complex will persist until S phase as CDH1 is slowly degraded by polyubiquitination from the APC, among other inhibitory regulation (Sivakumar *et al.,* 2015).

### 4.1.2 APC dysfunction and cancer:

A controversy currently exists as to whether the APC should be targeted for inhibition or activation as a therapeutic approach to treating cancer. This is due to the contradictory roles of the APC coactivators CDC20 and CDH1 in cancer development and progression. Overexpression of CDC20 has been identified as a prognostic marker for poor clinical outcomes for multiple unrelated cancer types including gastric (Ding *et al.,* 2014), breast (Karra *et al.,* 2014), colorectal (Wu *et al.,* 2013), prostate (Mao *et al.,* 2016), brain astrocytoma (Ding *et al.,* 2017) and pancreatic cancer (Chang *et al.,* 2012). Briefly, *CDC20* overexpression promotes malignant behaviour through overriding mitotic arrest induced by the SAC through excessive CDC20 accumulation in the cell, and promoting a dysregulated mitotic progression despite normal SAC activity (Bonauiti *et al.,* 2014; Pan *et al.,* 2004).

Conversely, impaired CDH1 activity promotes cancer progression, as it normally functions as a tumor suppressor. CDH1 haploinsufficient mice ($CDH1^{+/-}$) experience elevated instances of cancer development (Garcí-Higuera *et al.,* 2008). Furthermore, prolonged suppression of CDH1 results in acquired resistance to radiation therapy in mouse models of B cell lymphoma (Ishizawa *et al.,* 2017). CDH1 promotes multiple functions of the APC to act in a tumor suppressive manner (Wäsch *et al.,* 2010). Examples include the necessary role of CDH1 activity in the induction of stress responses (Simpson-Lavy *et al.,* 2008; Sudo *et al.,* 2001), checkpoint activation (Basserman *et al.,* 2008; Cappell *et al.,* 2016) and cell cycle regulation (Choudhurry *et al.,* 2016; Park *et al.,* 2008; Sigi *et al.,* 2009), mechanisms commonly disrupted in cancer. However, CDH1 also has oncogenic potential as it is capable of overriding SAC inhibition and inducing mitotic slippage (Nagai *et al.,* 2014; Park *et al.,* 2018; Toda *et al.,* 2012). Mitotic slippage refers to the process whereby, despite the activation of the SAC inducing mitotic arrest due to detection of DNA damage, the cell progresses into anaphase. Consequences of mitotic slippage include dysregulated proliferation, aneuploidy, and accrual of DNA damage (Crasta *et al.,* 2012; Ohashi *et al.,* 2015; Zhu *et al.,* 2014). The frequent correlation between accumulated APC substrates and more aggressive malignant tumor behaviors indicates that a generalized disruption of APC activity may occur during cancer progression (Lehman *et al.,* 2007; Zhang *et al.,* 2019). Impairment of APC activity may also arise through defects in the complex itself. Mutation or decreased expression of APC subunits *APC6, APC8, APC7,* and *APC16,* permits the unregulated proliferation of cells and promotes cancer development and progression (Wild al., 2018; Wang *et al.,* 2003), whereas deletion of other subunits causing severe loss of APC activity is lethal to even normal cells given its essential function. The disruption of APC activity has also been observed during the progression of canine lymphoma into an MDR state, where elevated APC activity corresponded with remission /chemosensitivity, whereas relapse correlated with APC dysfunction (Arnason unpublished).

### 4.1.3 Targeting APC as a therapeutic route to improving cancer responsiveness

Enhancing APC activity has generated great interest as a therapeutic possibility in the treatment of cancer. Currently, *in vitro* activation of the APC has been achieved through pharmacological inhibition of two SAC components, MAD2 and the TTK protein kinase. These agents have been designated MAD2 inhibitor 1 (M2I-1) and TTK protein kinase inhibitors (TTKi)

respectively. By targeting either subunit of the SAC, these agents induce their therapeutic effect through SAC impairment to elevate CDC20 availability, permitting its association with and activation of the APC (Kastl *et al.,* 2015; Wang *et al.,* 2019). *In vitro*, either agent alone or in combination with additional chemotherapies induced cell death and demonstrated potent anti-tumorigenic activity, with several TTKi treatments currently undergoing clinical trials (Maia *et al.,* 2018; Thu *et al.,* 2018; Wenger *et al.,* 2016). While much remains to be determined, it is thought that the therapeutically activated APC forces cancerous cells to undergo mitotic slippage, and incur the aforementioned consequences (Kastl *et al.,* 2015; Li *et al.,* 2019; Maia *et al.,* 2018; Thu *et al.,* 2018; Wengner *et al.,* 2016). Due to the role of TTK in regulating proper chromosomal segregation, there remains concerns about potential TTK inhibitor toxicity in proliferative non-cancerous cells (Martinez *et al.,* 2015). As of yet, there have been no agents capable of enhancing APC activity through directly binding the APC complex.

### 4.1.4 Direct activation of the APC through novel peptides

A series of novel peptides have been identified using a yeast 2-hybrid library as being capable of physically binding the APC, through either of the APC subunits APC5 or APC10. To investigate the interactions between these peptides and the APC, yeast strains harboring mutations in APC activity were utilized to examine the impact of peptide expression on APC phenotypes. Mutated APC in yeast can give rise to temperature sensitive growth, and marked decreases in yeast lifespan. Significantly, multiple peptides found to directly bind the APC were also capable of restoring the temperature sensitivity defect and of enhancing the replicative lifespans back to that of wild-type unmutated yeast strains. Due to the evolutionary conservation of the APC, including the APC5 and APC10 subunits, it is hypothesized these peptides will have a similar activating effect in mammalian cells (data unpublished).

Mammalian cells are not readily testable for lifespan or temperature sensitivity, such as is used to assess yeast APC mutants. Rather, direct and indirect measures of APC activity can be made in mammalian cells. The E3 ligase function of the APC can be semi-quantitatively measured through *in vitro* ubiquitination assays. Comparison of polyUb chain formation and chain abundance on known APC substrate proteins can identify the relative changes in APC E3 activity (Kraft *et al.,*2006). The impact of the novel peptides on APC activity can also be indirectly measured through the accumulation of APC substrates, and dysfunctional APC would accumulate normally degraded protein substrates, whereas activation of the APC would decrease protein abundance.

## 4.2 Materials and Methods

### 4.2.1 Creation of human peptide expression plasmids: Peptide Cloning

Random peptides were generated and then analyzed in a yeast 2-hybrid library to identify peptides capable of binding select subunits of the APC. Several peptides later demonstrated an ability to promote APC activity in yeast (data not shown), and were therefore cloned into

mammalian expression vectors was previously described in section 3.1. In order to promote small peptide stability, peptide-protein hybrids were constructed whereby the larger TrxA backbone provides stability to the smaller peptides of interest, and are unchanged from the protein expressed in yeast (only the promoter and selectable marker differ). From the proteins' N-terminus, a Haemagglutinin epitope tag (HA, sequence YPYDVPDYA) is fused in frame to the first thioredoxin subunit (TrxA, *Escherichia coli*) and separated from the second TrxA subunit by an in-frame peptide fusion (**Figure 4.1 A**). A second version of each peptide was created to include an SV40 nuclear localization sequence (NLS, sequence PKKKRKV) and a B42 transcriptional activating domain (enhances sensitivity in the yeast 2-hybrid library) to the peptide/protein hybrid; the remainder of the expressed peptide-protein hybrid was not altered otherwise (**Figure 4.1 B**).

Sanger sequencing was performed by The National Research Council (Saskatoon, SK) to confirm the proper in-frame insertion of the peptides into the backbone. It was noted that several peptides, in both the yeast and human peptide constructs, had a premature stop codon in the TrxA-peptide-TrxA DNA sequence through either a frame shift in the second TrxA subunit or via a stop codon within the peptide sequence itself (**Table 4.1**). The vector backbone used for human peptide expression is pcDNA3.1 (pcDNA), and contains CMV and T7 constitutive promoters. The pcDNA backbone was selected because of its constitutive expression permitted the observation of long-term affects of peptide expression on APC activity.

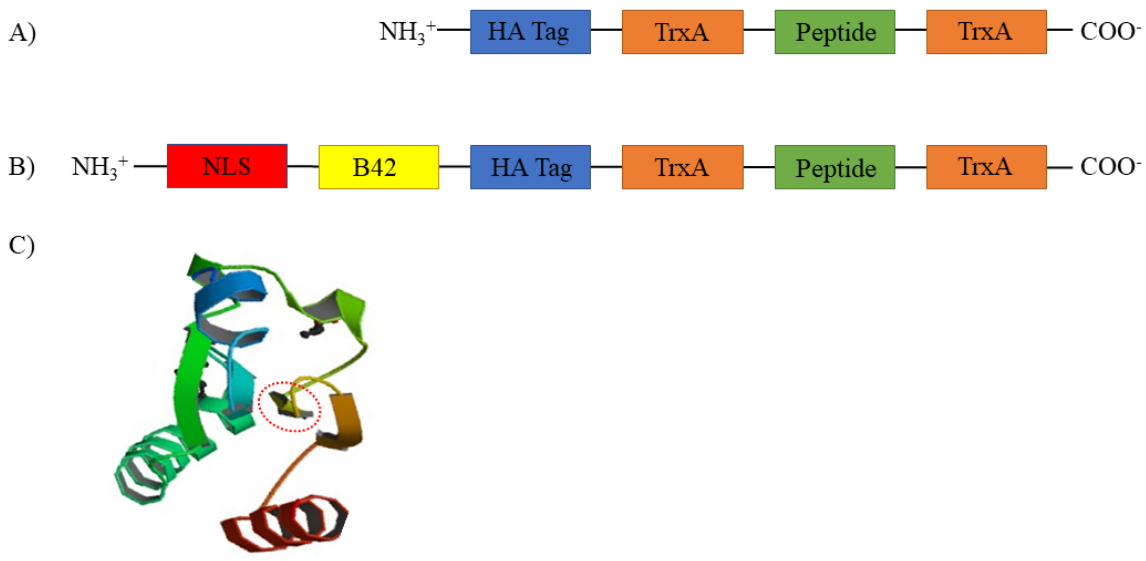



**Figure 4.1**. **Insert constructs inserted into the pcDNA backbone.** Sequences of the protein constructs inserted into pcDNA backbone. A) The insert without an NLS present. B) The peptide inserts with a SV40 NLS, and a B42 subunit to the backbone. C) Ribbon diagram represents the 2 domains of the *E. coli* thioredoxin protein, highlighting the position of the peptide insertions with a dotted red circle between the 2 domains (image adapted from PDB *Protein Data Bank*).

| Peptide Name | Predicted amino acid sequences of the peptides | Premature Stop | Bound APC Subunit from yeast 2-hybrid | Predicted Molecular Weight (kDa) |
|---|---|---|---|---|
| C1.8 | FCL* *GLSLLHLTFVPFGQL | Yes | APC5 | 6.0 |
| C2.4B | RMPQWWQWM | Yes | APC5 & APC10 | 7.6 |
| C3.1 | GRMLMTYLWYFMVLWVPRPWGPPLGLRGMWN*RFMNCPLVFLGPLSSGGEGFLYPWRL*LGESD | Yes | APC5 | 9.4 |
| C3.1B | VNGERWAP*SPNPRALCVL*GPLQSVGLFQHGNQGERWERLL | Yes | APC10 | 6.55 |
| C9.5 | CECLETETFHPITRHLIVPV | No | APC5 | 9.9 |
| C13.3 | GALKEVCICIVESVGGEVFS | No | APC5 | 10.9 |
| C43.4 | NGSSHNDLRVRRLTLISRLC | No | APC10 | 9.9 |

**Table 4.1. Characteristics of the peptides**. The amino acid sequences of the peptides, whether they induce a premature stop codon in the protein backbone, and the APC subunit bound. Premature stop codons in the DNA sequence are noted by an *.

A)

| Name | Gene ID | Start | Alignment with peptide | End | % Identity | % Positives |
|---|---|---|---|---|---|---|
| **Peptide C13.3** | | 1 | GALKEVCICIVESVGGEVFS | 20 | | |
| Cytokine SCM-1 beta precursor | NP002986.1 | 11 | GALKEVCICIVEGVGSEVFS | 26 | 56 | 56 |
| Tetratricopeptide Repeat Protein | XP016860458.1 | 740 | GALKEVCICIVETVGGEVFS | 747 | 88 | 87 |
| VitK Epoxide reductase complex | XP011514133.1 | 106 | GALKEFCICIVESVGGEVFS | 115 | 80 | 80 |
| Sodium/Hydrogen exchanger 9B2 Isoform | NP001287685.1 | 143 | GALKGVCXXCIVEVGGEVFS | 158 | 50 | 56 |
| 5-Oxoprolinase | NP060040.1 | 717 | GALKEVCICI--SVAGEVFS | 725 | 73 | 72 |
| Phosphatidylinositol 3,4,5-triphosphate/Rac exchanger 2 | XP011515914.1 | 1036 | GALKEVCVCIVESVGGEVFS | 1045 | 70 | 80 |
| AMPK α 1 subunit | NP001341964.1 | 329 | GALREVCFCVVESVGGEVFS | 339 | 64 | 72 |
| APC1 subunit | NP073153.1 | 146 | GALKEVCICILESVGGEVFS | 156 | 64 | 72 |
| Rab-21 Ras-related protein | NP055814.1 | 124 | GALKEICLCIVESVGGEVFS | 130 | 71 | 85 |

B)

| Name | Gene ID | Start | Alignment with peptide | End | % Identity | % Positives |
|---|---|---|---|---|---|---|
| **Peptide 43.4** | | 1 | NGSSHNDLRVRRLTLISRLC | 20 | | |
| CD14 Monocyte Differentiation antigen | NP000582.1 | 89 | NGSSHNDLRVRRLTLISRLC | 95 | 100 | 100 |
| | | 257 | NGSSHNSLRVRRLTLISRLC | 262 | | |
| Solute carrier family 6 member 2 X3 | XP016881489.1 | 167 | NGSSHNDLRVRRLTLISRLC | 182 | 63 | 62 |
| N-α acetyltransferase 25 | XP016875466.1 | 336 | NGSSHNDLRIRSLTL--RLC | 345 | 67 | 75 |
| | | 548 | NGSSHNDLRVRRLTLISRLC | 551 | 100 | 100 |
| CDK12 isoform14 | XP005257515.1 | 945 | NGSSHNDLRVRRLELISRLC | 952 | 88 | 87 |
| Eukaryotic initiation factor 3 | XP016879110.1 | 116 | NGSSHNDLRVRILTLVERMC | 135 | 55 | 65 |
| HERC 6 probably E3 Ub-ligase | XP06863822.1 | 266 | NGSSHNDLRVRR----SRLC | 273 | 67 | 66 |
| SUN-domain containing protein 2 | XP016884237.1 | 98 | NGSSHNDLRVRRLTLISRLC | 103 | 100 | 100 |
| | | 406 | NGSSVKEL--RRLTLISRLC | 414 | 55 | 63 |
| | | 335 | NGSSHNDLRVRRLTLVSRLC | 338 | 54 | 69 |
| Neuron navigator 2 isoform | XP011518756.1 | 690 | NGTAQSDLRIRRLTLISRLC | 702 | 54 | 69 |
| | | 964 | NGSSHNDLRVRRLTLISRLC | 967 | 100 | 100 |
| Serine-rich coiled-coildomain protein | XP016863690.1 | 7 | NGSSHNDLRVRRSTLVSRLC | 15 | 78 | 88 |

**Table 4.2. Sequence homologies of peptides to human proteins**. Sequence alignment of A) C13.3 and B) C43.4. Black indicates matching residues, red indicates mismatched residues, blue indicates conserved residues, and – indicates a sequence gap.

### 4.2.2 Cell Culture

The protocol was previously described in section 3.2

### 4.2.3 Western Blotting

The protocol was previously described in section 3.3. Imaging was performed with a VersaDoc (Bio Rad), and analyzed using VersaDoc software Quantity One (version 4.6.9).

### 4.2.4 Chemosensitivity Assays

The protocol to measure cell survival after exposure to cytotoxic chemotherapy was previously described in section 3.4. Two formats were used, a 6-well format and a 96-well format. The 6-well format exclusively utilized a 1 μM dose of doxorubicin (DOX) for 48 hours. Here, each trial consisted of 3 control wells and 3 treated wells, repeated 3 times. The 96-well format utilized a range of DOX doses from 0 to 1 μM, with 0.1 μM increments of DOX, for 48 hours. Each trial consisted of 4 control wells and 4 treated wells at each concentration, repeated 3 times.

### 4.2.5 Statistical Analysis

Unpaired t-tests were performed between the control cell line and individual peptide expressing cell lines to determine statistical significance. Statistical analysis was performed within the software of GraphPad Prism version 8.4.1. Confidence intervals were established by our collaborator Dr. Rana (University of Saskatchewan) utilizing a non-linear regression model with classical models of inference, a confidence interval was established to determine statistical significance in the dose-response curve, with an alpha level of 0.05 for hypothesis testing. Confidence intervals were generated in a R statistical environment (R Core Team, 2017) using the brms package (Bürkner, 2018).

## 4.3 Results

### 4.3.1 Determining the necessity for a Nuclear Localization Signal (NLS) sequence for peptide biological efficiency in human cells.

Because the APC complex and its E3 activity is reported to be exclusively localized to the nucleus (Sivakumar *et al.,* 2015), we queried if an NLS was required in our mammalian peptide expression constructs to enable a biological effect on APC activity. Therefore, two variants of each APC-activating peptide were produced for human cell expression. One version contained an in-frame NLS and one was expressed without, with no differences otherwise. Our preliminary screen to indirectly detect changes APC activity was performed utilizing western blotting of the APC substrates CDC20 and Cyclin B1 to determine if an NLS was essential to peptide function. Enhanced activity would result in increased degradation of the APC substrates via its E3 Ub-ligase activity. The results with peptides C1.8, C2.4B, C13.3 and C43.4 reveal that peptides without an NLS were more effective at reducing the accumulation of APC substrates (**Figure 4.2**). Additional peptides C3.1, C3.1B, and C9.5 were also examined and produced similar results (data not shown).

Therefore, experimentation with the NLS peptide variants was discontinued, and all following results utilized the peptide configuration without an NLS sequence, as noted in **Figure 4.1 A**.

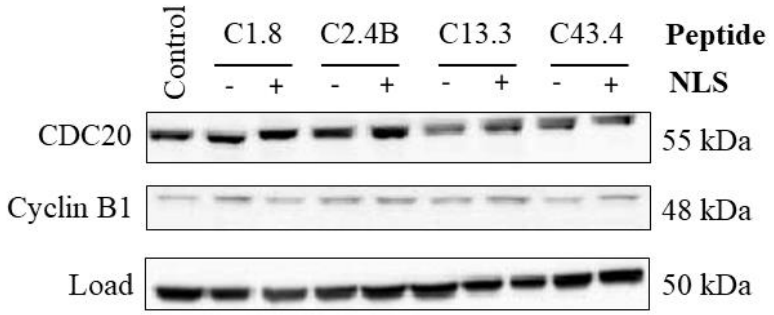



**Figure 4.2. Determining the necessity of an in-frame peptide nuclear localization signal on indirect APC activity.** Representative western blots of whole cell lysates prepared from 231-cells stably transfected with four different constitutively expressed peptides comparing the ability of peptides to induce APC activity with (+) and without (-) an N-terminal NLS. CDC20 and Cyclin B1 are known APC degradation targets. The load control is Tubulin. The control is a 231-cell line stably transfected with an empty pcDNA vector.

### 4.3.2 Cloning and detection of peptide expression constructs in an MDR human cell line

The peptide amino acids are embedded within the two lobes of the stable thioredoxin protein, which itself is 6.55 kDa in size **(Figure 4.1C**). Due to the existence of a premature stop codons in many peptide sequences (**Table 4.1**), the second globular TrxA- domain is missing from several peptides. Therefore, their molecular weights as predicted from sequencing range from 5.87 kDa to 10.88 kDa. Sequencing confirmed the in-frame cloning of the HA-TrxA-peptide sequences within the pcDNA vector, and revealed the expected molecular weight of each construct (**Table 4.1**).

The N-terminal HA epitope is expected to be detectable by anti-HA western blotting, to confirm expression after stable transfection. Initially, expression of C13.3 and C43.4 could be verified after stable transfection into 231 cells through HA blotting (**Figure 4.3**). However, these blots were later revealed to be non-specific background signaling. This was a result of an incorrect calculation of the full weight of the peptides, and the assumption that we were working with the full length bacterial thioredoxin (weight of 24 kDa), which when combined with most peptides would weigh around 30 kDa. This error was discovered from calculating the exact molecular weights acquired from the sequencing results and unfortunately, we have not been able to correctly establish peptide expression since due to the COVID-19 laboratory shutdown.

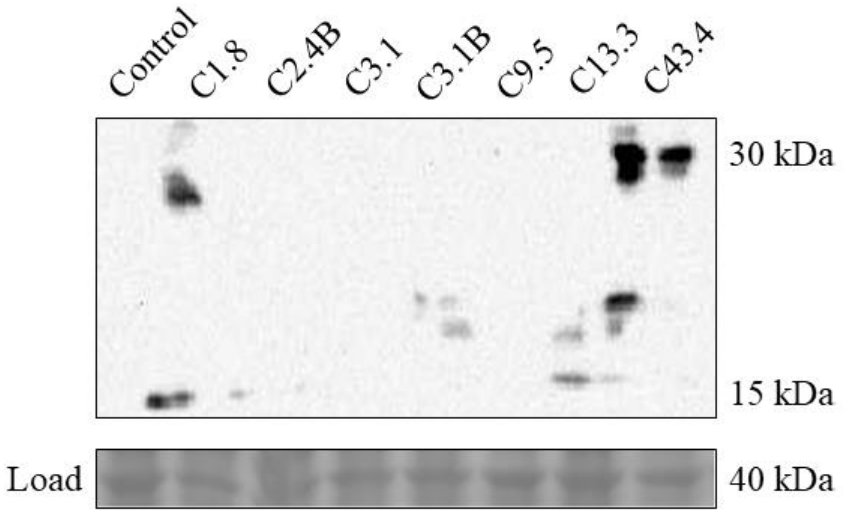



**Figure 4.3. Detection of peptide expression.** Whole cell lysates were prepared from eight 231 cell populations individually (stably) transfected with empty pcDNA vector (control) or one of seven constitutively expressed peptides, as indicated. A Western blot was performed from these cell lysates against the HA tag present at the N-terminus of each thioredoxin-peptide conjugate, with predicted weights between 6.0 and 10.9 kDa (**Table 4.1**). The 15 kDa marker represents the bottom/front of the acrylamide gel and is not resolved. The control is an empty pcDNA vector and does not have the HA epitope.

### 4.3.3 Indirect assessment of changes in APC activity upon constitutive peptide expression.

APC activity can be determined indirectly by qualitatively and quantitatively measuring the protein abundance of several known APC protein degradation targets, with the expectation that enhanced APC activity would be detectable as a decrease in target signal as compared to control. The degradation of substrates that simultaneously regulate APC activity may also yield insights into APC regulation. The degradation of Cyclin B1 by $APC^{CDC20}$ is necessary for the transition of the APC to CDH1 driven activity, as Cyclin B1-promoted phosphorylation inhibits $APC^{CDH1}$ binding (Sivakumar 2015). Remaining Cyclin B1 is then degraded by $APC^{CDH1}$ during mitotic exit simultaneously with CDC20. It is important to examine APC activity outside of the context of APC regulation, so we determined the relative abundance of three independent APC targets, Cyclin B1, HURP and CDC20 (**Figure 4.4)**. Peptide C43.4 produced the greatest decrease in two of the three APC substrates, and therefore presumably reflects a stronger increase in APC activity than any of the other peptides. In the presence of C43.4 versus control, a marked reduction in the protein abundance of Cyclin B1, HURP, and CDC20 was noted, a statistically significance difference for Cyclin B1 and HURP (49.9% [$p<0.0001$], 30.7% [$p=0.0047$] and 58.3% [$p=0.2372$], of the control, respectively). C13.3 also demonstrated increased APC activity, given the marked reduction of all

three targets as compared to control, reaching significance for Cyclin B1 (73.9% reduction: p<0.0001), and HURP (44.4% reduction: [p=0.0316], but not for CDC20 (49.3% reduction: [p=0.0702]. Other peptides were also capable of reducing substrate quantities, but in an inconsistent manner. C1.8 reduced CDC20 to 61.9% (p=0.0434), and C3.1 reduced HURP to 57.0% (p=0.0240) of control. Other peptides also approached statistically significant degradation of one or multiple substrates. The consistency of substrate degradation induced by C13.3 and C43.4 suggested that these two peptides provide enhancement to APC activity, with C43.4 producing the greater effect. The remaining peptides were excluded from further analysis.

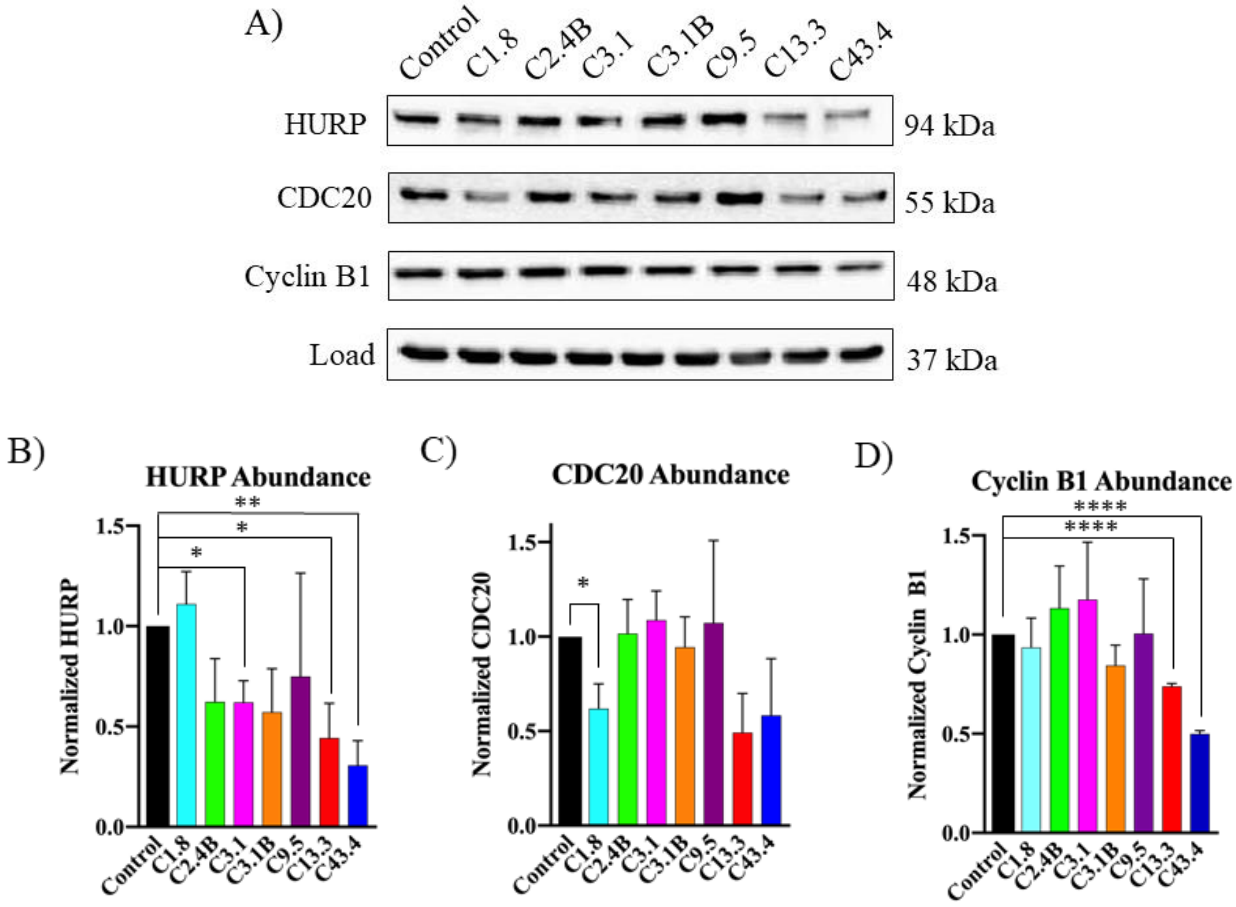



**Figure 4.4. Indirect assessment of APC activity changes with constitutive peptide expression.** (A) Qualitative representative Western blots of three APC protein degradation targets HURP, CDC20 and Cyclin B1 expressed by stable transfection in 231 cells. Quantitative representation of (B) HURP, (C) CDC20 and (D) Cyclin B1 protein abundance adjusted for the GAPDH load control, and normalized to the control [231 cell line stably transfected with an empty pcDNA vector], set to 1.00. n=3 biological repeats. Unpaired t-tests between control and individual peptides was performed to establish statistical significance; *=p<0.05, **=p<0.01, ****=p<0.0001. Error bars represent standard error of the mean (SEM).

### 4.3.4 Assessment of changes to MDR cell line chemosensitivity upon peptide expression.

Our observation that MDR cells lines and tumors had lowered APC activity than matched parental cell lines or control tissue had led us to hypothesize that increasing APC activity would restore the cellular death in response to chemotherapy (Arnason, unpublished) and restore chemosensitivity. The previous experiments (**Figure 4.4**) had revealed that both the C13.3 and C43.4 peptides were significantly able to increase the E3 ligase activity of the APC, at the level of target protein degradation. Next, we wanted to determine if the augmented activity of the APC induced by these two peptides would enhance the cell death in response to chemotherapy, measured as a reduced cell viability compared to the 231 MDR cell line without peptide expression. There are multiple ways to assess cell viability, and here we use the MTT assay, a colourimetric assay where only living cells can convert the assay reagent to a visible spectrum color (λ570) over a linear range. For each cell line/peptide combination, a dose of the chemotherapeutic DOX was delivered. After 48 hrs of treatment with DOX, cells are incubated with the MTT reagent; surviving cells metabolize the MTT into Formazan (purple) that can be quantitated through absorbance spectrophotometry after solubilization of the cells in DMSO.

Chemosensitivity assays were first carried out using our standard 6-well growth chamber format, allowing for triplicate repeats for each cell line with and without exposure to a near-lethal dose of 1.0 μM DOX. The control 231 MDR cell line was compared to 231 cells expressing either C13.3 or C43.4 (**Figure 4.5 A**). This dose caused an 83.5 % mortality rate (16.5% viability) in the control line which was minimally increased to 84.5% upon C13.3 expression (15.5% viability) and to 89.9% mortality rate upon C43.4 expression (10.1% viability).

Although the peptides appear to enhance cell death, the extensive killing of the control strain minimized our ability to see strong differences and therefore to make conclusions. Considering that the 1.0 μM DOX dosing was too potent to facilitate the detection of chemosensitivity differences between the peptides, we developed a DOX-dose-response MTT assay using a 96-well format. The DOX concentration increased by 0.1 μM increments from 0.1 μM to 1.0 μM (**Figure 4.5 B**) and demonstrated a clear separation between the chemosensitivity of 231 cell populations expressing peptide, versus without, and with C43.4 exhibiting a more potent cell killing than C13.3 at any given dose. The separation of differential chemosensitivities occurs at DOX concentrations of $\geq$0.5 μM. Beginning at the 0.5 μM dose, both peptides begin to diverge from the trend established by the control, and continue to enhance cellular death with the incremental dosage increases. C43.4 produces the strongest effect in elevating chemosensitivity, with an almost universally strong response to treatment at each dose level when compared to the control. A clear separation between 95% confidence intervals at DOX doses $\geq$ 0.5 μM between peptide expressing 231 cells and the control serves as a visual confirmation that the observed elevated response is statistically significant. (**Figure 4.4 C**).

The 1.0 μM dose, as performed in the 6-well format demonstrated itself an inappropriate measurement for cellular viability as the severe drop in viability between the 0.9 μM and 1.0 μM dose converged the divergent trends in chemosensitivity (**Figure 4.5 B)** between the peptide expressing cell lines and the control. The 1.0 μM dose demonstrated excessive potency, and overrode the impact of enhanced APC activity on chemosensitivity. By examining the lower doses

(0.5 μM-0.9 μM) presented in the 96-well format, a clear trend is present, that elevated APC activity induces an elevated response to chemotherapy. This supports our hypothesis, that enhanced APC activity reverses MDR behaviour.

These viability assays demonstrate that enhancing APC activity results in a reversal of MDR behaviour, as chemosensitivity was enhanced upon peptide expression. Both peptides elevated indirect APC activity, and the peptide that induced the greatest elevation in APC activity provoked the greatest augmentation of chemosensitivity. Together, this suggests an APC activity specific relationship between APC E3 function and MDR behaviour. This strongly supports the validity of our initial hypothesis, that enhancing APC activity can result in reversal of MDR behaviour and restoration of chemosensitivity.

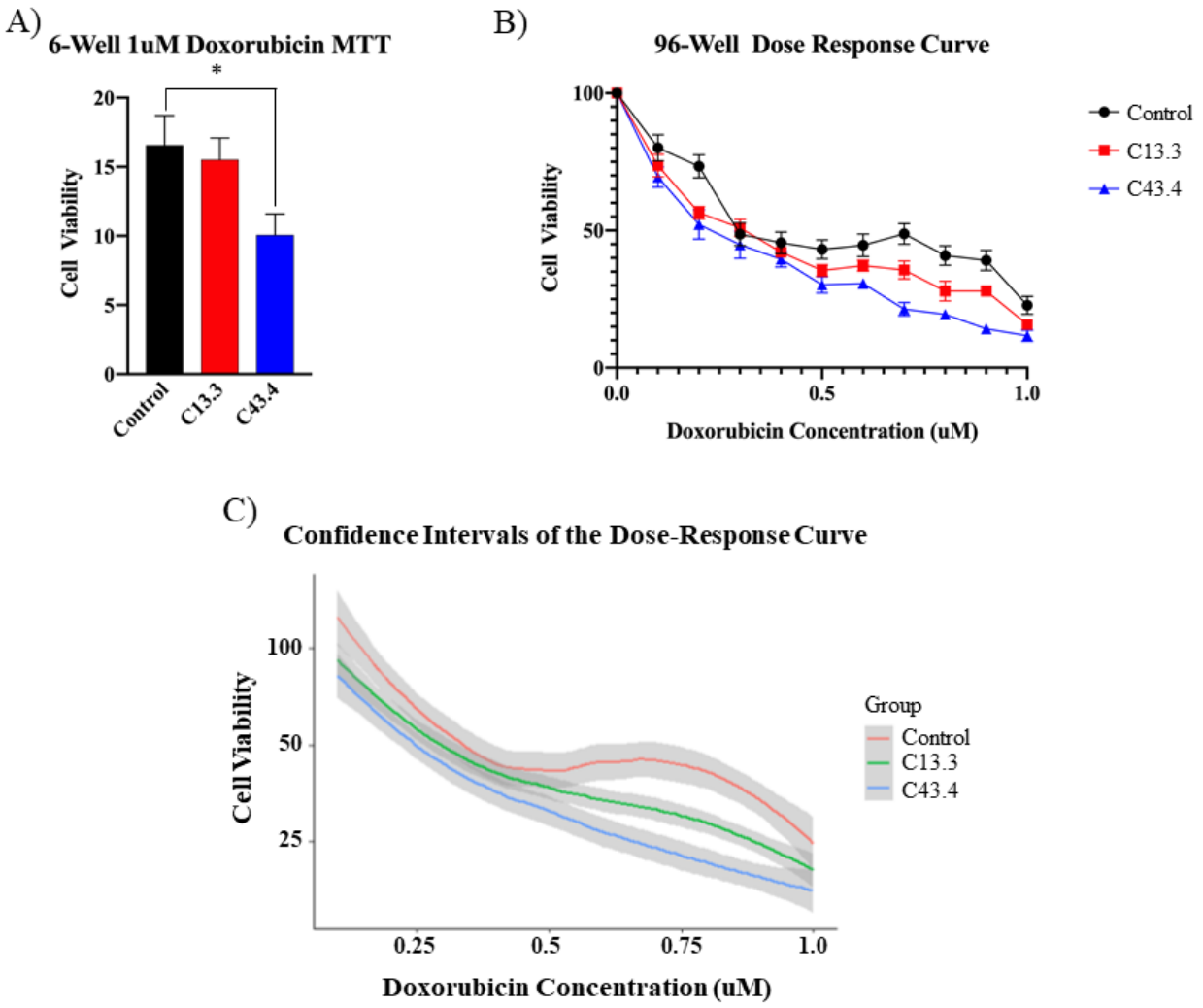



**Figure 4.5. Increased APC activity correlates with increases in MDR breast cancer chemosensitivity.** Changes in cellular viability due to peptide expression were measured using chemosensitivity assays using DOX as the cytotoxic agent and MTT as the reporter assay. A) 6-well chemosensitivity assays utilizing 1μM of DOX for 48 hours. Unpaired t-tests comparing individual peptide expressing lines to the control (a 231-cell line stably transfected with an empty pcDNA vector) were performed to establish statistical significance, n=9 technical repeats; *=$p<0.05$, error bars represent SEM. B) 96-well chemosensitivity dose-response assay ranging over DOX concentrations of 0 μM to 1 μM over 48 hours. n=10 technical repeats; error bars represent SEM. C) 95% confidence intervals over the dose response curve, generated as described in section 4.2.5.

## 4.4 Discussion

Due to previous observations correlating APC dysfunction with chemotherapy resistance/MDR behavior (Arnason unpublished), we hypothesized that enhancing APC activity will restore chemosensitivity in MDR cancer. Our novel approach to enhancing APC activity was to adapt APC-binding and -activating peptides discovered in yeast, into peptides capable of expression in human cell lines by cloning into human expression vectors. The yeast APC-activating peptides were discovered through 2-hybrid protein-protein interactions between APC subunits and a random peptide library, and were shown to be able to augment several discrete APC functions in yeast, including stress response pathways and longevity pathways These are well known APC function in yeast and easily assayed. Due to evolutionary conservation of the APC structure and function between *S. cerevisiae* and human, the peptides were predicted to also be able to activate the human APC. Longevity and stress pathway assessment in human cell lines are not established means of determining human APC activity, and instead we relied on indirect measures of APC E3-ligase activity, by monitoring target protein degradation.

To establish the optimal method of enhancing APC activity through peptide expression, each peptide of interest from our yeast studies was cloned into the expression vector pcDNA as one of two versions, either with an N-terminal NLS, or without (**Figure 4.1**). When tested for protein target degradation, peptide versions with the NLS unexpectedly reduced APC activity relative to their non-NLS counterpart (**Figure 4.2**). While we do not have a clear explanation for the loss of activity, it may be due to the NLS and B42 sequences inhibiting the binding capabilities of the peptides to the APC, possible arising because of the larger size of the protein backbone containing the NLS (22.48 kDa) versus, the backbone without the NLS (8.86 kDa). The additional amino acids in the NLS backbone may physically block the peptides from binding the APC. As part its regular function TrxA localizes to the nucleus, and this self-localization may assist TrxA-peptide localization, however this is unlikely a critical mechanism of peptide functioning. During previous research, commercially produced TAT2-peptide-FITC conjugates (University of Alberta, Edmonton AB, Canada) with no TrxA backbone have been synthesized. TAT2 (Trans-activating transcriptional activator 2) is a cell penetrating peptide derived from HIV, and upon conjugation with secondary molecules has been utilized as a drug delivery system (Mishra *et al.,* 2011). Fluorescein isothiocyanate (FITC) is a fluorescent molecule detectable through fluorescence microscopy (Chaganti *et al.,* 2018). After exogenously applying the TAT2-peptide-FITC conjugates to cancer cells, fluorescence of FITC indicated localization of the conjugates to the nucleus, therefore the peptides appear capable of self-localization to the nucleus without additional protein backbone (data unpublished).

Of the seven tested peptides (as depicted in **Table 4.1**), two (C13.3 and C43.4) demonstrated consistent elevation in APC activity across all three tested substrates (**Figure 4.3**). These were also two of only three peptides that did not contain a premature stop codon. Having two bounding thioredoxin domains surrounding the short peptide sequences may assist in peptide efficacy by having both peptide ends tethered and confined, providing a defined 3-dimentional structure to the peptide. This may produce a more effective conformation, rather than having a free

untethered C-terminus. Alternatively, it may be that the presence of the C-terminal region of thioredoxin provides a more stable protein hybrid product that is not rapidly degraded. *In vitro* studies with the exogenous application of C43.4 through the TAT2-C43.4-FITC conjugate demonstrated an ability to enhance chemosensitivity, indicating nuclear localization to activate the APC (data unpublished). The TrxA backbone, therefore, may not be essential to peptide function, however there is a noted difference in abundance between these approaches, namely exogenous application of a set quantity of peptide, versus continual endogenous expression.

Unfortunately, we were unable to verify the expression of any of the peptides after their stable transfection into the 231 cells (**Figure 4.3**). Despite this, there are clear biological differences noted in 231 cells and behavioural alterations arising from C13.3 and C43.4 expression compared to the empty vector control cells indicating they are likely expressed, despite the lack of confirmed expression by Western blot. Nonetheless, the purpose of experimenting with seven peptides was to identify the peptides most effective at elevating APC activity.

We do not currently know the mechanism by which the peptides elevate APC activity in mammalian cells, whether they behave similarly to each other, or have different mechanisms of action. They may act in identical ways just with different potencies, or their differences may reflect unique processes affecting APC function. We suspect the peptides directly bind specific APC subunits in human cells, in a similar manner to the yeast 2-hybrid global screen (data unpublished). This yeast screen found C43.4 through its binding to APC10, whereas C13.3 was identified though its binding to APC5 (**Table 4.1**). While both APC5 and APC10 subunits have highly conserved orthologs in human cells, we have yet to establish this direct physical interaction in mammalian cells.

Multiple potential mechanisms exist through which the peptides likely enhance APC activity, and given the discrete subunits bound by C43.4 and C13.3 in yeast, the specific mechanisms are likely distinct. As the APC is a multi-subunit complex, the interactions between subunits is a critical component for maintaining complex stability and therefore activity. APC5 plays a structural role in maintaining complex cohesiveness, and its degradation results in APC complex dissociation and loss of function (Chang *et al.,* 2014). Therefore, C13.3 may allosterically promote complex stability through binding to APC5, and therefore activity. APC5 also experiences numerous post-transcriptional events including phosphorylation, and ubiquitination sites (many with undefined functions) (Tran *et al.,* 2010; Zhang *et al.,* 2016). Therefore, binding of C13.3 to APC5 may also block inhibitory post-transcriptional modifications including inhibitory phosphorylation events or polyubiquitination (protecting from subsequent proteasomal degradation). APC10 does not experience significant post-transcriptional modifications (Zhang *et al.,* 2016) therefore, blocking post-transcriptional modifications is less likely to contribute to the elevated activity. APC10 functions to facilitate substrate recognition, and resides inside the binding pocket (Chang *et al.,* 2014). It remains possible that C43.4 enhances substrate recognition through APC10 associations. This could be achieved through allosterically altering the conformation of the binding pocket to permit more efficient substrate binding. These described mechanisms are based on the assumption that the peptides elevate the efficiency at which their

bound subunits perform their known functions. However, until further investigations are carried out to specifically investigating these mechanisms, the exact manner remains unknown.

The altered abundance of APC regulatory proteins which are also targeted for degradation (including those not characterized in this study, notably PLK1 (Zhang *et al.,* 2014) and CDH1 [Visintin *et al.,* 1997]) induced by peptide expression may also contribute to altered APC activity. The initial degradation of these regulatory proteins upon peptide activation of APC by peptide expression may create a feedback mechanism that modulates APC activity. The possible conclusions drawn from these decreased abundances of the APC activators Cyclin B1 and CDC20 (**Figure 4.4**) are tentative, but noteworthy. The degradation of Cyclin B1 is necessary for the binding of CDH1 to the APC (Kraft et al., 2003; Kramer et al., 2000), and therefore its elevated degradation is likely promoting the transition of APC activity between CDC20 and CDH1. This is supported by the degradation of CDC20, as it is solely performed by $APC^{CDH1}$ (Zhang *et al.,* 2014), indicating that the peptides are elevating $APC^{CDH1}$ activity. The degradation of CDC20 may prevent oncogenic behaviour, as CDC20 accumulation is a noted oncogenic behaviour (Chang *et al.,* 2012; Ding *et al.,* 2017; Ding *et al.,* 2014; Mao *et al.,* 2016; Wu *et al.,* 2013). However, as will be presented in **Figure 5.2**, NEK2A experiences elevated degradation from C13.3 but not C43.4 expression. As NEK2A is exclusively degraded by $APC^{CDC20}$ (Zhang *et al.,* 2014), this indicates that $APC^{CDC20}$ does experience an elevation in activity. The lack of C43.4 elevated degradation of NEK2A does not necessarily indicate that C43.4 does not promote $APC^{CDC20}$ activity, simply that it was not observed in this target.

The experiments presented here demonstrate that enhanced APC activity resulted in restoration of chemosensitivity, as shown by elevated cell killing of MDR cells in response to chemotherapy. Furthermore, the degree of restored chemosensitivity was in a manner proportional to the extent of APC activation (**Figure 4.5 A-C**).

It is notable that C13.3 and C43.4 contain sequence homologies to multiple proteins of interest despite their short sequences of 20 amino acids each. Several proteins have known biological functions that make the homology very interesting, and could be used to rationalized, in part, their ability to activate APC. Notably, when the peptide sequences were BLASTED for amino acid alignment again the yeast protein database, peptide C43.4 contains homology to the histone variant Htz1 and peptide C13.3 contains homology to Apc1, a critical subunit that is heavily post-translationally modified to regulate APC complex activity (Zhang *et al.,* 2016; data unpublished). General grouping of yeast proteins with homology to the peptides includes biological functions in proteasomal, stress response/metabolism, and DNA-chromosome interactions. The peptides were similarly BLASTED against human proteins, and identified several proteins that are known to have a diverse array of functions that may be potentially relevant to APC activity, and again demonstrate APC1 subunit similarity for peptide C13.3 (**Table 4.2**). The homologies between the peptides and these proteins may indicate as yet undiscovered interactions of the APC in yeast and their orthologues in mammalian systems.

Our observations that enhanced APC activity restores a degree of chemosensitivity leads to the question of the usefulness of this approach in cancer therapy. *In vitro* studies of cancer cell

responses when the APC was chemically inhibited and induced a severe toxic effect in healthy cells due to the suppression of APCs' essential functions (Zhang *et al.,* 2014). Here, our activation of the APC through peptide expression did not induce a toxic effect until paired with chemotherapy. The viability of cells exposed only to peptide expression was not significantly lower than baseline cellular viability, indicating that the peptides did not appear to produce an innately toxic effect. Others in the lab have confirmed this nontoxic effect of the peptides on whole animals (intraperitoneal peptide infusions into female mice), or when APC was stimulated using commercial APC activators (MAD2 inhibitors) *in vitro*. This supports the safety and usefulness of the APC activation approach for future cancer therapeutic studies as healthy cells may be relatively unaffected by the peptide treatment.

When considering the usefulness of these peptides for direct cancer therapy, there are several potential issues and limitations unique to protein and peptide therapies. In *in vivo* models, the exogenous application of peptide-conjugates may induce an immunological response and prevent effective treatment. Also, the proteins may not have a sufficiently long half-life to confer a meaningful serum concentration, or there may be a failure to correctly target the malignancies at therapeutic concentrations. Our experiments utilized endogenous expression of the peptides which would be impractical in a patient. Despite these obvious concerns, the TAT2-C43.4-FITC peptide conjugate has demonstrated an *in vitro* capability to induce chemosensitivity in a similar manner to our observations. Furthermore, Gabby Mercer (MSc candidate) in our lab has successfully xenografted C13.3 and C43.4 expressing 231 cells *in vivo* in mice. Significantly, the tumors demonstrated a dose-dependent inhibition of tumor growth over a 21-day experiment after a single intraperitoneal peptide dose, indicating that the peptides promote anti-tumorigenic effects *in vivo*. If necessary, to circumvent the issues related to protein-based therapies, after establishing the exact mechanism of peptide action on the APC, pharmacological mimetics of the peptides could be identified and synthesized. Despite these challenges, the principle of enhancing APC activity as a therapeutic model remains a promising option for cancer treatment.

# Chapter 5. Mechanisms Relating APC Activity to Multiple Drug resistance Behaviour

## 5.1 Introduction

### 5.1.1 Multiple drug resistance (MDR) behaviour and the APC

During cancer development and progression, the individual cancer cells experience alterations to their behaviour including metastasis, altered proliferative patterns, and acquire MDR. Based on selective pressures, these behaviours may become adopted throughout the population (Gerlinger *et al.,* 2012). MDR behaviour is characterized as the resistance of the cancer population to multiple, mechanistically unrelated therapies including those not used for treatment (Holohan *et al.,* 2013; Housman *et al,*.2014). MDR behaviour arises through impaired apoptosis, stress response pathways, dysregulated mitosis, and genomic instability (Austin Doyle *et al.,* 1998; Choi 2005; Fernald and Kurokawa, 2013; Lee *et al.,* 2011; Miyashita and Reed, 1993; Zhu *et al.,* 2014). Proper functioning of the APC promotes the activation of stress responses, and promotes a regulated mitotic cycle, thereby combating multiple mechanisms of MDR behaviour. To investigate the link between APC dysfunction and MDR development, a series of novel peptides were identified in a yeast 2-hybrid library as being capable of binding and enhancing APC activity. Upon subcloning of these peptides into mammalian expression vectors, they were stably transfected into the innately MDR breast cancer cell line 231. Two peptides were identified that both promoted enhanced APC activity and cytotoxic sensitivity of the cells to the chemotherapeutic agent DOX (**Figure 4.4 & 4.5)**. Here we will investigate the mechanistic underpinnings reversing MDR behaviour upon enhancement of APC activity.

### 5.1.1 Mitosis and genomic instability

The APC is named for its first recognized biological function, that of promoting the regulated progression of mitosis, specifically through mitosis into anaphase. It has been long recognized that the APC and the associated SAC are required to be functional to prevent cells harboring DNA damage or chromosomal defects from passing through this cell cycle stage and expanding through division. Cancer is well known to arise due to mutations and chromosomal defects, and impairments in APC function is known to correlated with cancer development. Furthermore, more aggressive cancers (e.g., treatment resistant or metastatic) appear to correlate with greater degrees of APC dysfunction. This has been observed indirectly through the accumulation of APC substrates in malignant compared to benign tumors (Lehman *et al.,* 2007). A gene network has also been identified whose expression is correlated with an elevated expression of *CDC20* including APC regulators and substrates, indicating generalized APC dysfunction during cancer progression (Zhang 2019). There has also been an association in canine lymphomas experiencing impaired APC activity as they transition into an MDR state (Arnason, unpublished).

The specific role that APC plays in controlling mitotic progression and promoting genomic integrity has been the focus of multiple studies and has revealed elegant molecular mechanisms involved in coordinating the cell cycle with quality checks, as well highlighting the defects that arise when APC is dysfunctional (Visintin *et al.,* 1997, Kramer *et al.,* 2000; Izawa *et al.,* 2015; Cappell 2018; Garcí-Higuera *et al.,* 2008; Sivakumar 2015). If we consider the initiation of mitosis as our starting point to discuss the role of APC activity within the cell cycle, then even prior to chromosomal alignment during metaphase there are key regulatory steps occurring. Here, the APC is inhibited by the SAC through sequestration of the APC co-activator CDC20 (Pan and Chen, 2004). Upon chromosomal attachment to microtubules the SAC releases CDC20 permitting its binding to the APC and forming the active $APC^{CDC20}$ complex. The APC then functions through its Ub-ligase function to target and degrade key proteins to enable regulated chromosomal segregation. One of the first proteins targeted for polyubiquitination by $APC^{CDC20}$ is Securin, triggering its proteasomal degradation, and allowing for chromatin separation to their respective poles. Specifically, Securin allosterically inhibits the protein Separase, which cleaves the kleisin subunit of cohesin, which holds sister chromatids together (Gligoris *et al.,* 2014; Lin *et al.,* 2016). The APC simultaneously targets Cyclin B1 for gradual degradation which ultimately results in the replacement of the co-activator CDC20 ($APC^{CDC20)}$ with CDH1 ($APC^{CDH1}$) in coordination with the cell cycle, until only $APC^{CDH1}$ remains it is and this complex that functions throughout mitotic exit and G1 (Castro *et al.,* 2005; Kramer *et al.,* 2000; Visintin *et al.,* 1997). Specifically, degradation of Cyclin B1 prevents CDK1 from placing the inhibitory phosphorylation onto CDH1 which would normally inhibits CDH1 binding and incorporation into the APC complex. Therefore, after Cyclin B1 degradation by $APC^{CDC20}$, CDH1 gradually replaces CDC20 to form $APC^{CDH1}$, initiating the second phase of APC activity. During mitosis, the early primary role of $APC^{CDH1}$ consists of preparing the cell for a regulated mitotic exit, again through substrate degradation (Kramer *et al.,* 2000; Visintin *et al.,* 1997).

There are several points where APC dysfunction could contribute to new cancer development or progression into more aggressive forms. A dysregulated mitosis is one of the principle mechanisms leading to the chromosomal instability that occurs in cancer cells, and is a primary driver of cancer progression (Crasta *et al.,* 2012; Gerlinger *et al.,* 2012; Lee *et al.,* 2011; Ohashi *et al.,* 2015; Zhu *et al.,* 2014). Due to the sustained chromosomal instability during cancer development, cancer cells often experience sustained SAC activity, as chromosomes fail to properly align during metaphase. This prolonged SAC activity, is referred to as mitotic arrest (Dalton *et al.,* 2007; Quignon *et al.,* 2007). In healthy cells, mitotic arrest typically resolves as chromosomes eventually successfully align, in which case the SAC becomes inactivated and the cell progresses through mitosis due to normal APC activity. Alternatively, prolonged mitotic arrest can instead result in apoptosis (Riffell *et al.,* 2009). Interestingly, in cancer cells a third outcome of prolonged SAC activation is mitotic slippage where cancer cells override SAC inhibition and the cell (inappropriately) progresses through mitosis (Bonaiuti *et al.,* 2018; Jiang *et al.,* 2003; Toda *et al.,* 2012; Park *et al.,* 2018). Consequences of mitotic slippage include dysregulated cell proliferation, chromosomal mis-segregation, resistance to microtubule poisons, and accumulation

of generalized DNA damage (Anand *et al.,* 2003; Crasta *et al.,* 2012; Jiang *et al.,* 2003; Riffell *et al.,* 2009; Ohashi *et al.,* 2015; Sudo *et al.,* 2004; Zhu *et al.,* 2014).

There are multiple mechanisms through which cancer cells can induce mitotic slippage. First is the over-accumulation of CDC20 which may arise through its overexpression or impaired degradation. The overabundance of CDC20 overrides SAC activity, as the SAC lacks the capacity to sufficiently sequester the excess CDC20 protein, and the APC becomes activated despite continued chromosomal misalignment (Bonaiuti *et al,* 2018; Pan and Chen, 2004). Mitotic slippage can also be induced by the second coactivator CDH1. ATP depletion during a prolonged mitotic arrest results in the loss of inhibitory phosphorylation on CDH1, permitting its binding to the APC before CDC20, and inducing chromosomal segregation (Nagai *et al.,* 2014; Park *et al.,* 2018; Toda *et al.,* 2012,).

### 5.1.2 Stress response pathways

The impairment of stress response pathways by cancer is multi-faceted and provides advantages to tumor growth and survival through the inhibition of apoptosis, and promotion of proliferative pathways (Fernald and Kurokawa, 2013; Gardai *et al.,* 2004; Kinoshita *et al.,* 1997; Vander Heiden and DeBerardinis, 2017). Yeast and mammalian studies have identified conserved stress response networks that experience frequent alterations in cancer, and multiple interactions have been identified between the APC and these pathways. In yeast, genetic interactions between the APC and Forkhead 1 and 2 (orthologues to mammalian FOXO transcription factors) highlighted their intersecting role in inducing stress responses and resultant apoptosis (Malo *et al.,* 2016; Postnikoff *et al.,* 2012). FOXO proteins are a family of transcription factors that intensify stress responses through expression of key genes/proteins, including Death Receptor 4/5, $p27^{Kip1}$, Bim, and Bcl-6 (Greer and Brunet, 2005; Roy *et al.,* 2010; Shoeb *et al.,* 2013). The elevation of FOXO activity (particularly FOXO3A) in cancer has demonstrated anti-tumorigenic effects and induces apoptosis *in vitro* (Dey *et al.,* 2015; Roy *et al.,* 2010; Shoeb *et al.,* 2013; Thayyullathil *et al.,* 2011). In mammalian systems, the FOXO family is regulated through AKT phosphor-signaling, which is a downstream effector of multiple growth factor stimulated pathways (Pros *et al.,*2013). In response to nutrient sensing and growth factor signaling, AKT activity is promoted by the PI3K pathway and inhibits stress responses while promoting cell proliferation through its phosphorylation of multiple proteins including the transcription factor FOXO3A (Brunet *et al.,* 1999). Accordingly, activation of FOXO3A through the inhibition of AKT has demonstrated therapeutic efficacy in cancer cytotoxicity (Dey *et al.,* 2015; Roy *et al.,* 2010; Shoeb *et al.,* 2013; Thayyullathil *et al.,* 2011). AKT activity is also directly associated with MDR behavior in malignant cells, and is specifically associated with an elevated abundance of the MDR makers, MDR-1 and MRP, members of the ABC drug efflux transporter family (Zhou *et al.,* 2013). AKT activity also promotes the vesicle mediated transport of the ABC transport protein BCRP for surface expression (Goler-Baron *et al.,* 2008). The inhibition of AKT has demonstrated therapeutic potential, overcoming MDR behaviour, inducing cell arrest and apoptosis, and is under clinical

investigation for therapeutic potential in cancer treatment with multiple AKT inhibitors in clinical and preclinical trials (Bartholomeusz *et al.*, 2012; Konopleva *et al.*, 2014).

AKT is endogenously negatively regulated through protein phosphatase 1 (PPP1) which acts as a tumor suppressor protein. PPP1 dephosphorylates the activating (phospho)-sites on AKT to inhibit its activity (Thayyullathil *et al.,* 2011). PPP1 itself is regulated by phosphorylation through multiple proteins including the mitotic kinase NEK2A (Helps *et al.,* 2000; Rellos *at al.,* 2007,), and the cyclin dependent kinases CDK1 and CDK2. (Blethrow *et al.,* 2008; Guo *et al.,* 2002). CDK1 activation is driven by Cyclin B1 during G2 and M to promote mitotic initiation and progression, and CDK2 is driven by Cyclin A2 to promote the G1/S and G2/M cell cycle transitions.

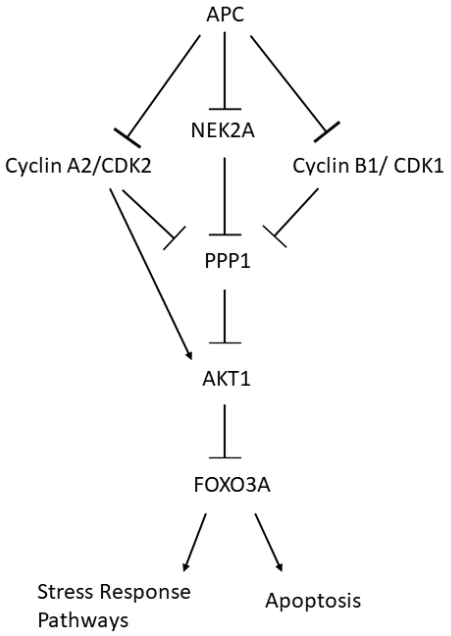



**Figure 5.1. Schematic of the cellular pathway connecting APC E3 activity to the activation of stress response pathways.** The APC substrates Cyclin A2, Cyclin B1, and NEK2A are inhibitors of stress responses through inhibition of PPP1 activity. Each substrate performs an inhibitory phosphorylation on residue T320 of protein phosphatase 1-alpha catalytic subunit (PPP1CA, Blethrow *et al.,* 2000; Guo *et al.,*2002; Helps *et al.,* 2000; Rellos *et al.,* 2007) to inhibit PPP1 activity. PPP1 is then prevented from dephosphorylating AKT1 at sites Threonine450 (T450) and Serine473 (S473), promoting AKT1 activation (Thayyullathil *et al.,* 2011). Active AKT1 performs inhibitory phosphorylation on Serine253 (S253) of FOXO3A (Brunet *et al.*, 1999). Elevated degradation of the APC protein substrates Cyclin B1, Cyclin A2, and NEK2A would hypothetically reduce the inhibition of PPP1, triggering dephosphorylation of AKT1 to permit FOXO3A activity and enhance stress response and apoptotic pathways. Pointed arrows represent activation of the downstream proteins, blunt arrows represent inhibition of the downstream proteins.

## 5.2 Materials and Methods

### 5.2.1 Cell Culture

The protocol was previously described in section 3.2.

### 5.2.2 Western Blotting

The protocol was previously described in section 3.3. In 231 cell lines, we assessed relative apoptosis rates by measuring Poly (ADP-ribose) polymerase (PARP) cleavage, after inducing cytotoxic stress via incubation in the presence of either 0.5 μM or 1.0 μM doxorubicin (DOX) for 48 hours prior to cell lysis. To determine general DNA damage abundance, histone H2A phosphorylation (γH2A.X) was measured. Cells were passaged for 1 month prior to lysis to permit observable alterations to DNA damage and accumulation.

### 5.2.3 Fluorescence Microscopy of DNA and Mitotic Figures

The protocol was previously described in section 3.5. Asynchronous 231 cells were fixed in 4% paraformaldehyde for 10 minutes before permeabilization with 0.5% Triton X-100 for 10 minutes. Cells were then stained with the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI) and visualized using fluorescence microscopy. Criteria for mitotic catastrophe includes visible failure to properly align chromosomes during metaphase, lagging chromosomes during anaphase, micronuclei formation, and asymmetric nuclei formation. A minimum of 250 cells were sampled for each population for each repeat.

### 5.2.4 Kinome Array

This protocol was previously described in section 3.6, and the array itself was performed by collaborators in the Dr. Napper lab (University of Saskatchewan), whereas the analysis and pathway construction was performed by us. The kinome array was performed as previously described (Määttanen *et al.,* 2013). Briefly, it involved immobilized pre-selected peptides known to be phosphorylated by discrete kinases. Using cell lysates prepared from treated cell lines (our control [empty pcDNA], C13.3, and C43.4 cell lines) and applying them to the peptide array allows for phosphorylation to occur where possible, and quantitation to be performed. The kinome array was constructed to investigate a wide range of peptide targets from multiple signaling pathways to reflect many cellular behaviours. Changes in phosphorylation between the control cell line and the peptide cell lines greater than +/- 1.5-fold change (FC) were considered significant. Note that this is a smaller difference than that considered significant for gene expression arrays where +/- 2 FC is often the cut off for significance differences. Changes in phosphorylation between the control cell line and the peptide cell lines between +/- 1.2 and 1.5-fold were considered moderate changes. The associated pathways established from the kinome data were assembled with assistance from KEGG PATHWAY database (https://www.genome.jp/kegg/pathway.html)

### 5.2.5 Statistical Analysis

The protocol for unpaired t-testing as performed in western blotting and imaging experiments was previously described in section 3.2.5. Statistical analysis for the kinome array was performed as described previously (Määttanen *et al.*, 2013). Briefly, a chi-square test examined technical variability and phosphorylation sites with variability above an acceptable threshold (as determined by the chi-square test) were excluded from further analysis. T-tests were then performed for each phosphorylation site/peptide target between the control and APC-activating-peptide expressing samples. As the chi-square test has already screened for technical variability, a p value threshold of p=0.2 was utilized to reduce the likelihood of false negatives.

## 5.3 Results

### 5.3.1 Assessment of the AKT-FOXO Stress response pathway as a result of altered APC activity

Guided by previous studies demonstrating the genetic interactions between the APC and yeast Fkh proteins to induce stress responses (Malo *et al.*, 2016; Postnikoff *et al.*, 2012), the impact of enhanced APC activity on the evolutionarily conserved activating FOXO protein phosphorylation signal was examined. To investigate the role of augmented APC activity in regulating the AKT/FOXO3A pathway, two 20-amino acid novel peptides (dubbed C13.3 and C43.4), that were previously established to enhance APC activity (**Figure 4.4**) were cloned into a mammalian expression vector and stably transfected into the innately MDR cell line, MDA-MB-231 (referred to as 231). To explore the pathway(s) connecting APC activity to FOXO3A activation, three degradation substrates of the APC known to interact with the AKT-FOXO3A pathway were examined for their stability as APC activity was manipulated. The substrates analysed included Cyclin A2, Cyclin B1, and NEK2A (**Figure 5.1**), and we observed that there were variable degrees of enhanced target protein degradation upon peptide expression. C43.4 reduced Cyclin B1 abundance to 49.9% ($p<0.0001$), and Cyclin A2 to 70.6% ($p=0.3625$) of the control, while failing to noticeably reduce NEK2A accumulation (**Figure 4.3 A, D and Figure 5.2**). C13.3 reduced Cyclin B1 abundance to 73.9% ($p<0.0001$) and NEK2A abundance to 67.3% ($p= 0.1208$) of the control, while failing to reduce Cyclin A2 levels (**Figure 4.3 A, D and Figure 5.2**). The variability in substrate degradation between the peptides highlights that the augmented APC activity induced by peptide expression invokes some degree of preference for substrate targeting.

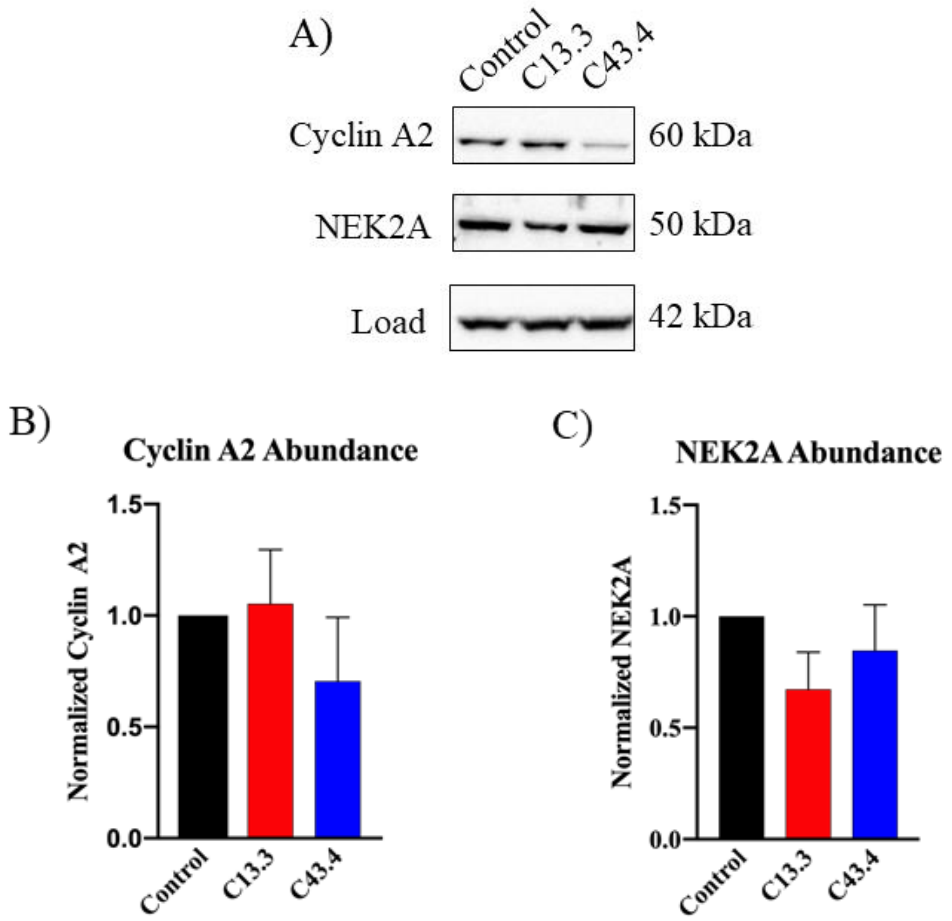



**Figure 5.2. Enhanced APC activity increases degradation of APC substrates known to inhibit FOXO3A signaling and stress responses.** A) Qualitative representative Western blots of the APC protein substrates NEK2A and Cyclin A2. Quantitative representation of B) Cyclin A2, C) NEK2A, protein abundance is adjusted to the actin load control, then normalized to the control value of 1.0 (231 cell line stably transfected with an empty pcDNA vector. n=3 biological repeats. Unpaired t-tests between control and individual peptides were performed to establish statistical significance; Error bars represent SEM.

We predicted that the observed decrease in FOXO3A inhibitory proteins upon APC activation would result in increased pathway activity. The impact on the signalling activity of the AKT/FOXO3A pathway was directly tested by searching for altered phosphorylation levels of three key regulatory phospho-targets within the stress response pathway, both increases and decreases depending on whether they were activating or inhibitory modifications.

The impact of peptide C43.4 (and presumably the activation of the APC) on the inhibitory p-PPP1CA T320 was to reduce the signal to 68.6% (p=0.0583, **Figure 5.3 A, B**) indicating increased activity of PPP1α and subsequent activation of the FOXO-mediated stress pathways. p-AKT1 S473 abundance was also modestly reduced to 83.9% (p=0.0749, **Figure 5.3 A, C**)

indicating a mild reduction in activity demonstrated by a consistent reduction in phospho-protein, rather than the magnitude of the change. Phosphorylation of FOXO3A at S253 was reduced to 54.6% (p=0.0028) of control (**Figure 5.3 A, D**), indicating a significant elevation in FOXO activation. This demonstrates that C43.4 appears capable of inducing stress response pathways through the activation of FOXO3A transcriptional activity, presumably through enhanced APC activity.

p-PPP1CA T320 was reduced by C13.3. expression to 65.4% (p=0.0284) indicating an elevation in PPP1α activity. Notably, p-AKT1 S473 was different for the C13.3. peptide, as it was elevated to 226% (p=0.1535) of the control. Despite the apparent elevation in AKT1 signaling (and the expectation of FOXO repression resulting therein), p-FOXO3A S253 was also reduced 58.8% (p=0.0031) by C13.3, and to a similar extent to that observed for C43.4 (**Figure 5.3 A, D**). Together, this suggests that both C43.4 and C13.3 expression are capable of elevating FOXO3A activity and stress response pathways, yet the mechanisms or the differences between them are not currently understood.

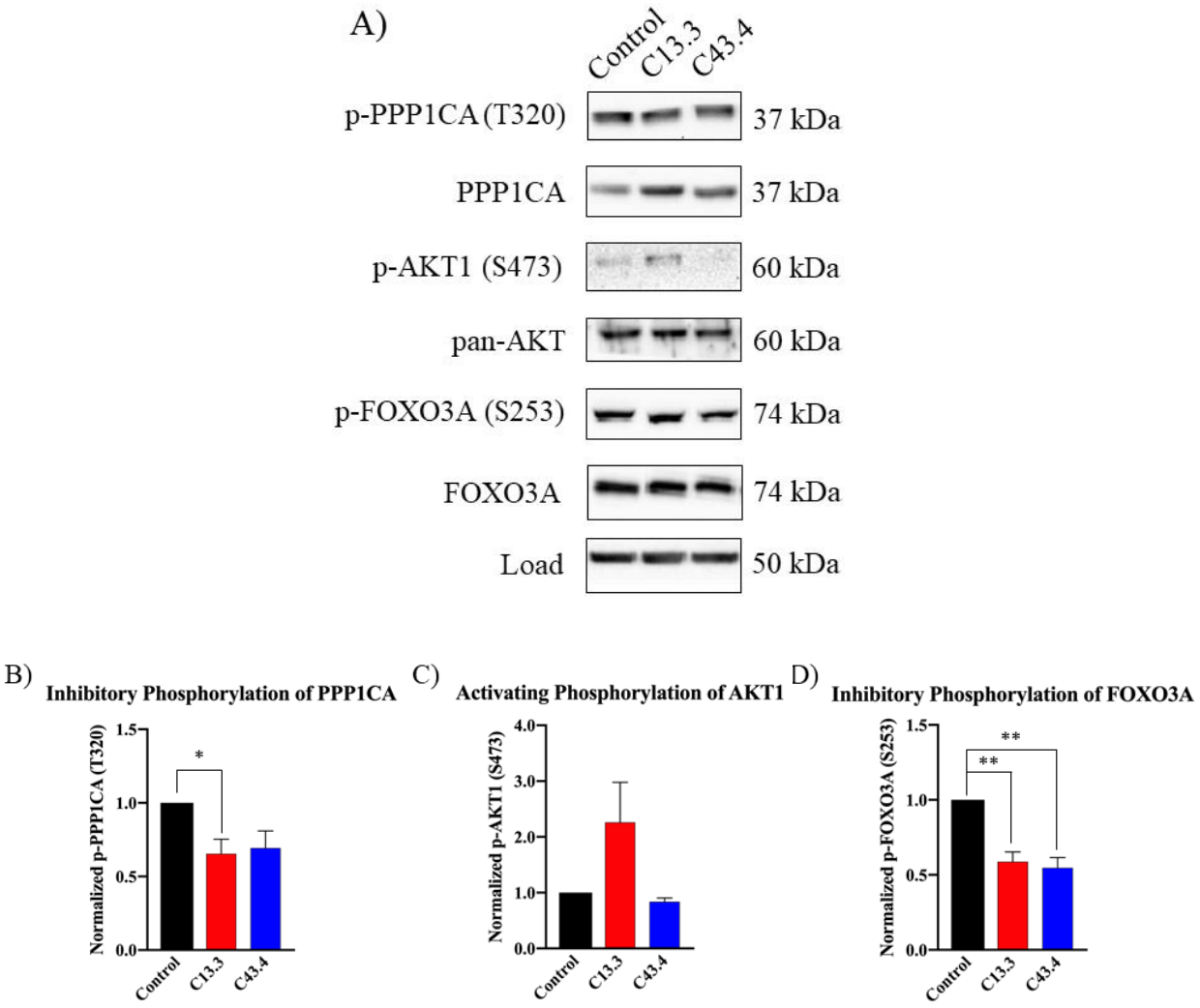



**Figure 5.3. Enhanced APC activity promotes stress responses through FOXO3A signalling.** A) Qualitative representative Western blots of the stress response pathway relating APC activity to the AKT-FOXO3A signaling pathway. Quantitative representation of B) p-PPP1CA (T320), C) AKT1 (S473), D) p-FOXO3A (T320), E) p-AKT (S473), F) and p-FOXO3A (S253). Phospho-protein abundance is adjusted to total protein and Tubulin load control, then normalized to the control value of 1.0 (231 cell line stably transfected with an empty pcDNA vector n=3 biological repeats. Unpaired t-tests between control and individual peptides were performed to establish statistical significance; *=$p<0.05$, **=$p<0.01$. Error bars represent SEM.

We also considered how to assess for changes in nutrient and energy signalling pathways in response to APC activity (via peptide expression), as these pathways are well established to also converge on FOXO to modify its activity downwards (rich nutrients inhibits stress signaling and apoptosis) or upwards (energy depletion augments stress response pathways and promotes cell survival and longevity) (Greer and Brunet, 2005). Cell culture conditions are designed to support optimal cell growth by providing excess nutrients and energy. Therefore, cellular networks would be shifted to suppress stress responses and apoptosis, and instead promote proliferation. We were therefore interested to determine the effect that stressful growth conditions would have on our 231 MDR breast cancer cells, and the impact that APC activation would make to the pathway balance.

Low glucose media (1000 mg/L D-glucose compared to the 4500 mg/L D-glucose in standard media) was used as a means of mimicking combined nutrient and energy starvation. To examine the effect of enhanced APC activity on stress response networks in response to metabolic stresses 231, cells were cultured in low glucose media for 5 days. Using these nutrient/energy stress conditions, we observed significant reductions in the inhibitory FOXO3A phosphorylation site at S253 with both peptides. When compared to the control (empty pcDNA), peptide C13.3 reduced this phosphorylation to 39.9% ($p=0.0295$) and C43.4 reduced this phosphorylation to 43.8% ($p=0.0063$, **Figure 5.4**). The elevation in FOXO3A activity upon peptide expression under low glucose conditions is a more significant enhancement than that observed under standard "rich" growth conditions (**Figure 5.3 A, D**). This indicates that enhancing APC activity elevates the stress responses of MDR cancers to both cytotoxic and noncytotoxic stresses and may contribute to their chemoresponsiveness as shown by enhanced cell death.

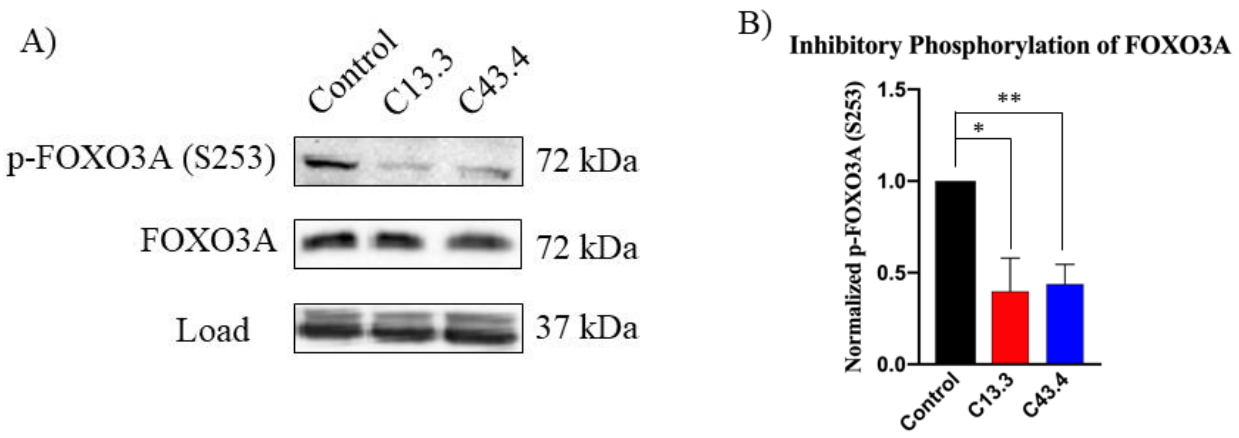



**Figure 5.4. Enhancing APC activity sensitizes the cell to metabolic stresses by promoting FOXO3A activity.** 231 cells were cultured in low glucose media for 5 days to induce a stress response through energy deprivation. A) Qualitative representative Western blot of total FOXO3A and phopshorylated-FOXO3A (Serine253) from whole cell lysates after the energy stress. B) Quantitative representation of p-FOXO3A protein abundance adjusted for the GAPDH load control and normalized to the control (231 cell line stably transfected with an empty pcDNA vector), set to 1.00. n=3 biological repeats. Unpaired t-tests between control and individual peptides were performed to establish statistical significance; *=$p<0.05$, **=$p<0.01$. Error bars represent SEM.

Common biomarkers of MDR cancer behaviour are the elevated abundance of several members of the ABC transport protein family including BCRP, MDR-1, and MRP-1. These proteins act as non-specific drug efflux pumps, protecting cancer cells from chemo- and molecular therapies (Choi, 2005). Driven by NEK2A activity, AKT promotes the expression of multiple ABC transporters through AKT's downstream targets, including both PIM1 and NFκB proteins (Zhou *et al.,* 2013). AKT also directly promotes the vesicle mediated transport of BCRP to the cell surface to promote drug efflux (Goler-Baron *et al.,* 2008). Therefore, the observed alterations in NEK2A abundance (**Figure 5.2 A, C**) and AKT activity (**Figure 5.3 A, C**) driven by enhanced APC activity may decrease ABC transporter abundance and reverse MDR behaviour. C43.4 reduced BCRP abundance to 76.1% (p=0.1824) and MDR-1 to 82.0% (p=0.1791) of control (**Figure 5.5**). C13.3 was unable to significantly alter BCRP levels and elevated MDR-1 to 149% (p=0.2388) of the control, although inconsistency in the C13.3 lysates prevents this from being a conclusive elevation (**Figure 5.5**). As the reductions in MDR marker abundance from C43.4 are not statistically significant, nor the observed change in protein accumulation large, it is difficult to draw a conclusion to the biological relevance of this apparent alteration. If these reductions induced a biological impact it could contribute to the enhanced chemosensitivity seen in C43.4 expressing 231 cells (**Figure 4.5**). The potential ability for C43.4 to reduce ABC transporter abundance

supports the idea that C43.4 is impairing AKT1 activity (**Figure 5.3 A, C**) and thereby causing alterations in cellular behaviours.

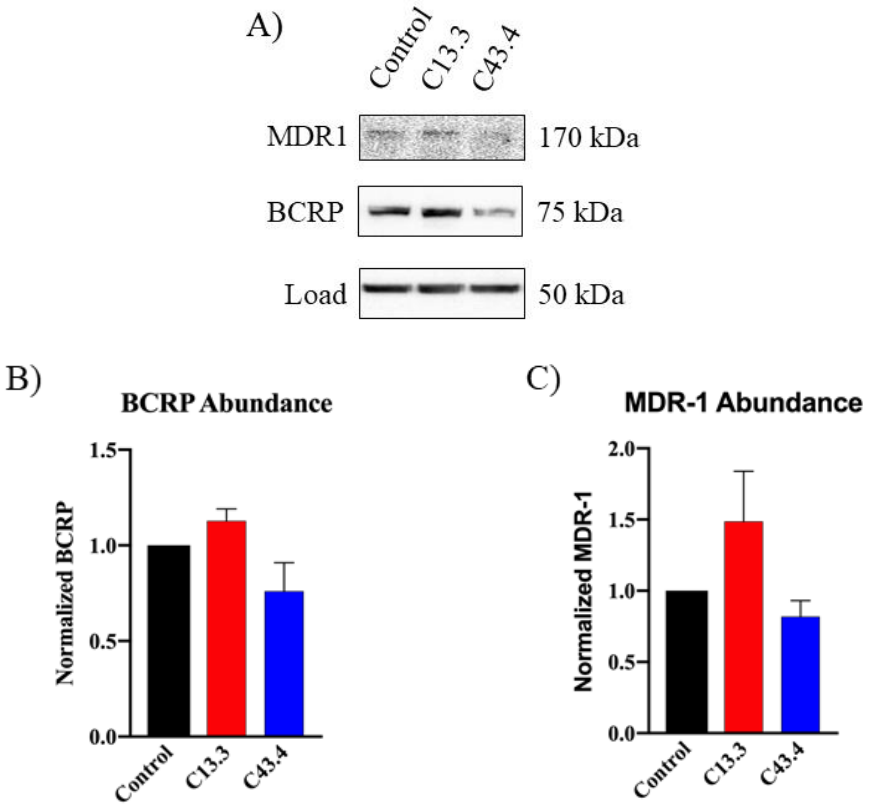



**Figure 5.5. Enhancing APC activity potentially lowers the abundance of classic MDR protein biomarkers, BCRP and MDR-1.** A) Qualitative representative western blot of BCRP and MDR-1 from whole cell 231 lysates. Quantitative representative Western blot of B) BCRP and C) MDR-1. Quantitative representation of B) BCRP, and C) MDR-1 protein abundance adjusted for the tubulin load control and normalized to the control (231 cell line stably transfected with an empty pcDNA vector), set to 1.00. n=3 biological repeats. Unpaired t-tests between control and individual peptides were performed to establish statistical significance. Error bars represent SEM.

### 5.3.2 Enhanced APC activity promotes apoptosis

We investigated if the observed changes in the activity of the stress response pathway upon APC activation pathways resulted in a shift in the balance between apoptosis versus cellular proliferation in our MDR breast cancer cell populations, as fully activated stress pathways do promote the fidelity of cellular checkpoints and result in apoptosis of damaged cells (DeBardinis *et al.,* 2008; Gong *et al.,* 2018). Our previous observation that APC activation restored chemosensitivity/cell death in our inherently MDR cell line suggested to us that the altered FOXO3A phosphorylation may ultimately demonstrate increased apoptosis. To detect apoptosis,

multiple complementary markers were investigated under both cytotoxic and non-cytotoxic conditions.

First, to examine the capability of the cytotoxic chemotherapy agent DOX to induce apoptosis in MDR cells with or without enhanced APC activity, 231 cells were exposed to 0.5 μM and 1.0 μM concentrations of DOX for 48 hours. The relative abundance of the apoptotic marker PARP (and its respective cleavage products) was done through western blotting. PARP protein undergoes predictable cleavage events during apoptosis initiation, generating a 89 kDa and a 24 kDa fragment (Nicholson *et al.,* 1995). At 0.5 μM, we were unable to detect PARP cleavage. This suggests that at lower concentrations of DOX, the observed decrease in cellular viability (**Figure 4.5**) may be a result of cells beginning apoptosis, but not completing the process. At 1.0 μM PARP cleavage (cPARP) products were detected with expression of both peptides. We have previously noted that MDR cancer cells experience an enhanced cell death response to chemotherapy upon peptide-induced APC activation, and this experiment suggests that the cell death in MDR cancer is through promotion of apoptotic pathways.

Second, apoptotic pathway biomarkers, Caspase 8 and 3 activity, were investigated in the 231 cells with and without peptide activation of the APC. We used a caspase reporter kit capable of simultaneously measuring both (Abcam). Caspase 8 activity is indicative of early activation of the extrinsic apoptotic pathway (Olsson and Zhivotovsky, 2011), whereas Caspase 3 activity is indicative of later stages when the cell is completing apoptosis, and is activated by both the intrinsic and extrinsic apoptotic pathways (Olsson and Zhivotovsky, 2011). The reporter kit provides a more sensitive measure of apoptotic activity than western blotting for PARP; however, the caspase kit was unsuitable for examination of apoptosis after DOX treatments, as there is no method of equilibrating surviving cell numbers between the different sample treatments that differs due to peptide expression, whereas surviving cell numbers can be adjusted through lysate loads prior to western blotting.

Expression of both C13.3 and C43.4 were pro-apoptotic in the 231 cell lines as compared to control when examined at the 1.0 μM DOX dose, with C43.4 providing the more significant elevation (**Figure 5.6 A-C**). This correlates with C43.4 inducing the more significant enhancement in chemosensitivity (**Figure 4.5**), indicating that inducing apoptosis is likely the main mechanism driving the reversal of MDR behaviour. The activity of caspases 3 and 8 was also elevated upon peptide expression demonstrating the rate both of initiation and progression of apoptosis under non-cytotoxic conditions is elevated through enhanced APC activity (**Figure 5.6 E, F**). However, unlike the observed alterations in PARP cleavage, C43.4 did not provide a significant elevation in activity over C13.3. Combined, both approaches of measuring apoptotic activity demonstrate a clear trend that enhancing APC activity results in a shift toward apoptosis and cellular death, even if the individual measurements failed to reach the traditional definition of significance.

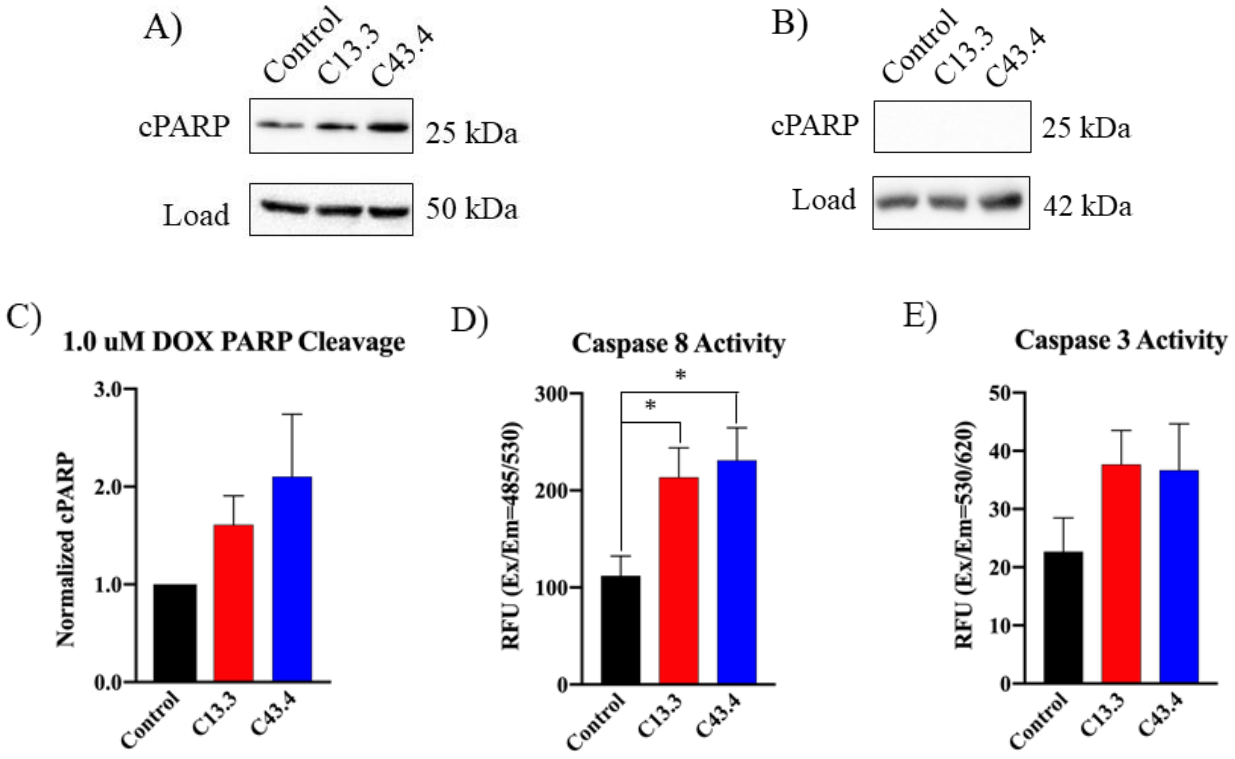



**Figure 5.6. Apoptotic pathways are increased upon peptide-induced APC activation.** Qualitative representative Western blot of PARP protein cleavage from whole cell lysates of 231 cells A) exposed to 1.0 μM DOX for 48 hours B) exposed to 0.5 μM DOX for 48 hours. C) 1.0 μM DOX exposure induced PARP cleavage, with the abundance adjusted to load control (Tubulin) and normalized to the control (231 cell line stably transfected with an empty pcDNA vector), set to 1.00. n=3 biological repeats. Activity of D) Caspase 8 and E) Caspase 3 was quantified using a caspase activity kit (Abcam). Caspase activity is expressed as relative fluorescence unit (RFU), a measurement of fluorophore activity. Excitation (Ex) and emission (Em) spectra are expressed as Ex/Em for their respective wavelengths. n=3 technical repeats. Unpaired t-tests between control and individual peptides were performed to establish statistical significance. Error bars represent SEM, *=$p<0.05$.

### 5.3.3 Enhanced APC activity induces mitotic dysregulation and genomic damage

A hallmark characteristic of malignant cells is dysregulation of the cell cycle, particularly mitosis. As mentioned previously, a dysregulated mitosis is a key promoter of cancer progression, inducing chromosomal mis-segregation, DNA damage, and unregulated proliferation (Crasta *et al.,* 2012; Ohashi *et al.,* 2015; Riffell *et al.,* 2009; Zhu *et al.,* 2014). To examine the impact of mitotic dysregulation upon APC activation by the peptides, we examined the change in prevalence of mitotic errors (mitotic catastrophes) in control 231 cells versus peptide-expressing 231 cells.

These are genotoxic events that occur as a result of mitotic dysregulation which would normally result in cell death in healthy cells and are readily visible under fluorescent microscopy after nuclear staining (Caruso *et al.,* 2011). We questioned if APC activation would result in fewer mitotic errors due to increased apoptosis of abnormal cells even before entry into mitosis. However, we also acknowledged the possibility of increase mitotic errors occurring due to APC activity forcing grossly defective cells through mitosis, resulting in mitotic catastrophe and genomic damage, that may then result in enhanced sensitivity to the chemotherapeutic DOX, as it compounds the damage accrual.

Asynchronous cells were fixed to simultaneously arrest a representative MDR cell population at their current point in the cell cycle. Nuclei, chromosomes, and mitotic figures are readily visualized by fluorescent microscopy upon DAPI staining. We counted the percent of cells within each population residing in mitosis, as well as those experiencing a visibly abnormal mitotic progression/mitotic catastrophes. Although typically fatal in healthy cells, cancer cells experience an elevated tolerance to these defects and are frequently more prevalent in malignant populations (Birkbak *et al.,* 2011; Crasta *et al.,* 2012; Dodgson *et al.,* 2016; Torres *et al.,* 2010).

In 231 control cells (pcDNA vector without peptide-expression), we observed 2.92% of cells undergoing a regulated mitosis, and 3.98% experiencing a mitotic catastrophe, a relatively high rate but consistent with the aggressive MDR phenotype of this cancer line (**Figure 5.6 B**). The expression of either of the APC-activating peptides resulted in a significant increase in cells experiencing mitotic catastrophes. C13.3 elevated catastrophes by 69.1% ($p<0.0001$), and C43.4 elevated catastrophes by 37.2% ($p=0.0077$, **Figure 5.6 B**). Both peptides also induced elevation in the number of cells imaged undergoing a regulated mitosis, although the specific implications of this phenomenon are unclear. The significant rise in mitotic catastrophes induced by both peptides indicates that enhancing APC activity leads to further dysregulation of the mitotic cycle, contrary to our initial hypothesis.

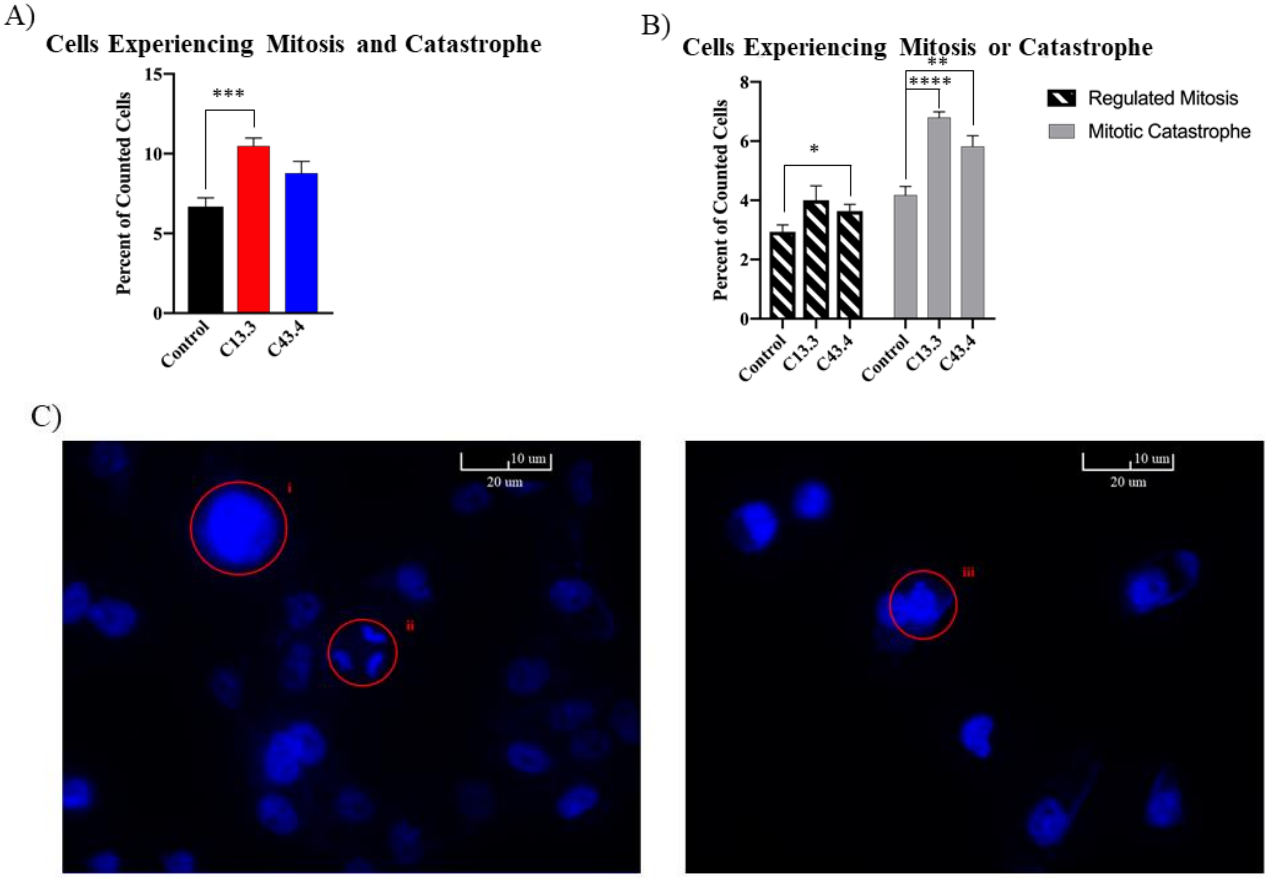



**Figure 5.7. Peptide-enhanced APC activity in MDR cells increased the mitotic catastrophe rate independent of cytotoxic or metabolic stresses.** A) The percent of imaged cells residing in either regulated mitosis, or experiencing a mitotic catastrophe in an asynchronous population (combined). Unpaired t-tests established statistical significance between the APC-activating peptide expressing cell lines versus control (231 cell line stably transfected with an empty pcDNA vector); n=5 biological repeats error bars represent SEM. B) Asynchronous cells from **(A)** subdivided into those experiencing a regulated mitosis versus cells experiencing mitotic catastrophes. Unpaired t-tests established statistical significance between the peptide expressing cell lines and the control; n=5 biological repeats, error bars represent SEM. C) Examples of mitotic catastrophes captured by fluorescent microscopy. Highlighted cells include a tetraploid cell (failure to undergo cytokinesis, [i]), a tri-directional anaphase [ii], and micronuclei formation [iii], Caruso *et al.,* 2011; Quignon *et al.,* 2007).

To explore if altered mitotic regulation resulted in the accumulation of DNA damage, we used phosphorylated H2A histone family member X abundance (γH2A.X, a histone variant recruited to sites of double strand breaks) as a quantitative marker for relative DNA damage accumulation. 231 cells were passaged for a month, either with or without peptide expression, before lysis to reach a steady state of γH2A.X accumulation in these cell populations. We observed that the cell population expressing APC-activating peptide C43.4 induced a significant accumulation of γH2A.X compared the 231 cells without APC activation, with an increase in γH2A.X abundance of 172% (p=0.0532) over the control (**Figure 5.8**). This is consistent with our detection of elevated rates of mitotic catastrophes, as errors in mitosis commonly contribute towards the accumulation of DNA damage (Crasta *et al.,* 2012, Ohashi *et al;* 2015, Zhu *et al.,* 2014). Overall, enhancing APC activity by C43.4 increased detectable DNA damage, and would this contribute to chromosomal instability. C13.3 did not significantly alter γH2A.X accumulation (**Figure 5.8**). It is possible that the elevation in mitotic catastrophes observed in C13.3 are inducing elevated necrosis (a mechanism not observed through caspase or PARP cleavage), thereby clearing the more significantly damaged cells from the population.

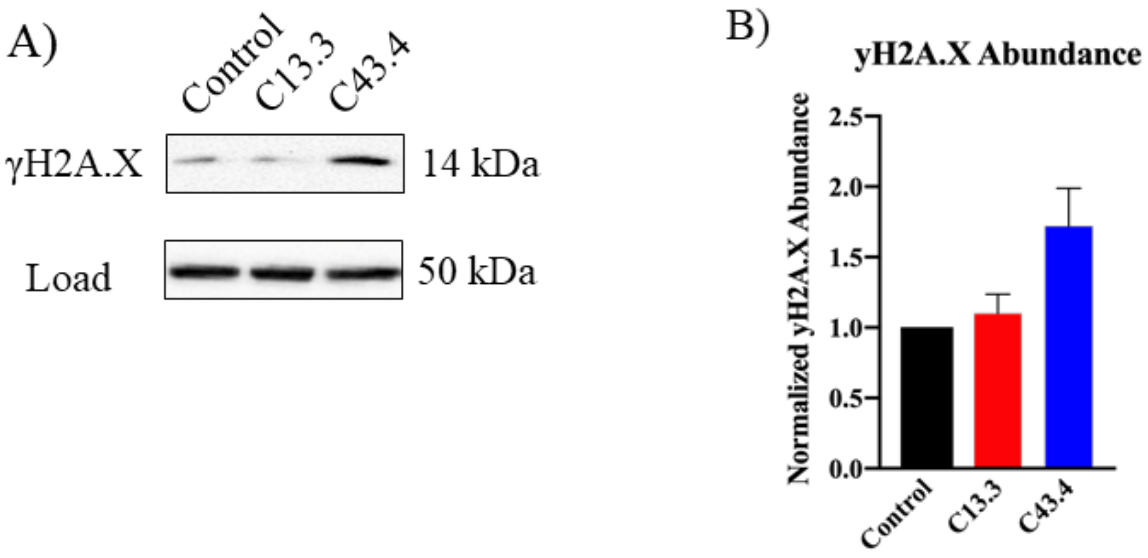
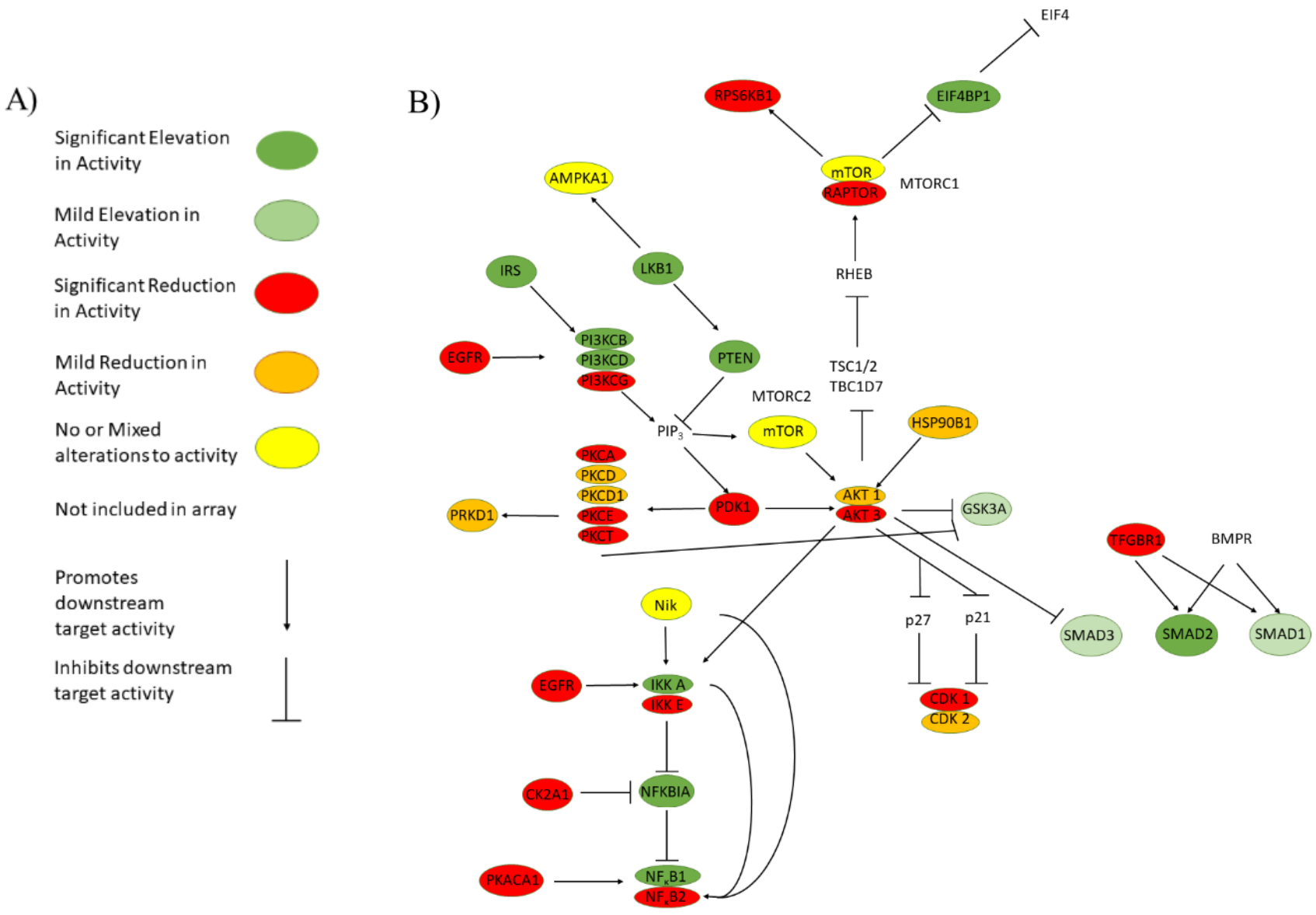



**Figure 5.8. Dysregulated mitosis provoked by enhanced APC activity elevates DNA damage accumulation.** A) Qualitative representative Western blot of γH2A.X using whole cell lysates of 231 cells passaged for one month in the presence or absence of APC-activating peptides. B) Quantitative representation of A) γH2A.X protein abundance adjusted to the Tubulin load control and normalized to the control (231 cell line stably transfected with an empty pcDNA vector), set to 1.00. n=3 biological repeats. Unpaired t-tests between control cell population and those expressing the individual peptides were performed to establish statistical significance. Error bars represent SEM.

### 5.3.4 Impact of Peptide expression on signaling pathways as measured by a Kinome Array

A kinome array measures the phosphorylation levels of specific phosphorylation sites on peptides immobilised onto an array. The phospho-peptides are often custom selected to represent proteins across multiple signalling pathways of particular interest. A comparison between the relative phosphorylation levels of two or more treatments to that of control establishes the changes in protein activity that occur between treatments as regulated through protein phosphorylation. Our array was created for another breast cancer experimental system, but a gracious donation of unused arrays provided us with a unique opportunity. In this case, our comparison was between 231 cells expressing the APC-activating peptides C13.3 and C43.4 versus empty control vector, pcDNA. While previous sections divided the results into C13.3 and C43.4, here we observed that the effect that each peptide had was similar, typically only varying in the degree of impact, and are therefore considered and presented together (raw data for individual peptides can be found in Appendix A.2 and A.3 for C13.3 and C43.4 respectively).

Two signaling networks are described in depth due to known influences with/on APC and their presence of the microarray; PI3K/AKT signaling and mitogen activated protein kinase (MAPK). In general, peptide expression resulted in detectable alterations in protein phosphorylation consistent with the inhibition of both PI3K/AKT and MAPK signaling pathway. Importantly, both pathways promote proliferation and cell growth, while simultaneously inhibiting stress responses and apoptosis, such that inhibition of these pathways could enable apoptosis and perhaps explain the reversal of chemoresistance in these cells (Foukas *et al.,* 2010; Kinoshita *et al.,* 1997; Miller *et al.,* 2011; Tokunaga *et al.,* 2006; Roy *et al.,* 2010). Also significant is the consistency with our previous experiments that demonstrated an elevation in stress responses and apoptosis upon APC-activating peptide expression.


A)
Significant Elevation in Activity
Mild Elevation in Activity
Significant Reduction in Activity
Mild Reduction in Activity
No or Mixed alterations to activity
Not included in array
Promotes downstream target activity
Inhibits downstream target activity
B)
EIF4
RPS6KB1
EIF4BP1
mTOR
RAPTOR
MTORC1
AMPKA1
LKB1
IRS
RHEB
EGFR
PI3KCB
PI3KCD
PI3KCG
PTEN
TSC1/2
TBC1D7
MTORC2
mTOR
HSP90B1
PIP3
PKCA
PKCD
PKCD1
PKCE
PKCT
PRKD1
PDK1
AKT 1
AKT 3
GSK3A
TFGBR1
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NFκB2

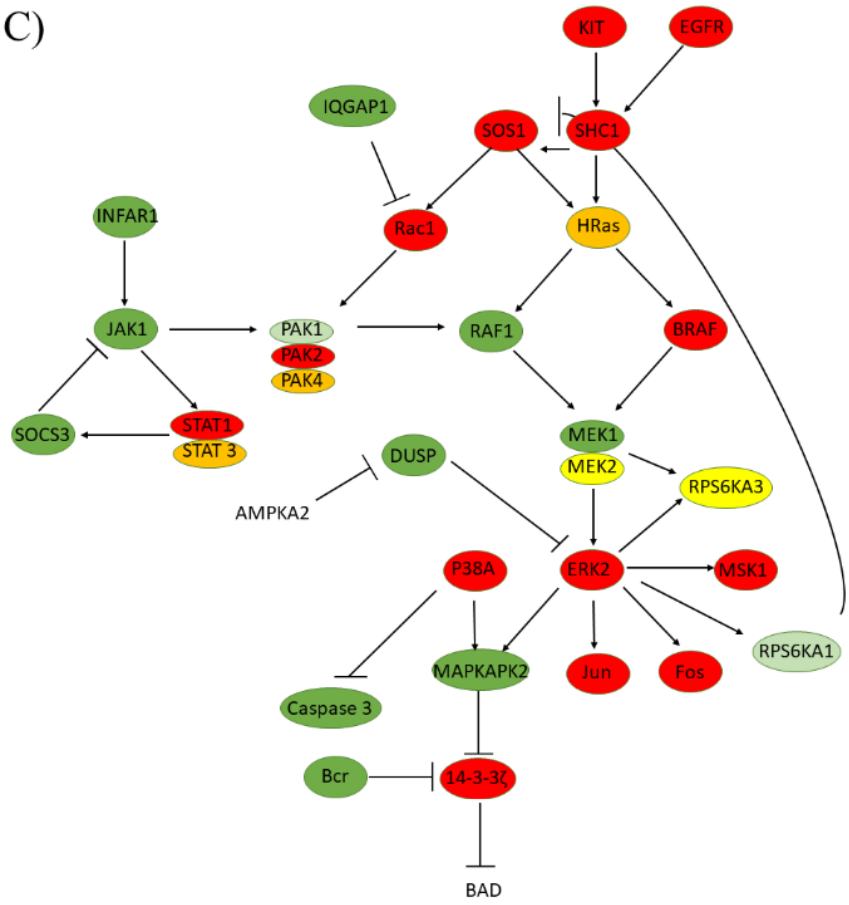


**Figure 5.9. AKT and MAPK Pathway alterations with elevated APC activity were revealed by a kinome array.** Protein activity as determined by the relative changes in phosphorylation on known activating and inhibitory sites. Phosphorylation abundance changes greater than a 1.5-fold indicates a significant change, between 1.2 and 1.5 are considered mild changes, and phosphorylation changes less than 1.2-fold are considered to be no change. Proteins that experienced phosphorylation changes that would both promoted and impaired activity were considered to have mixed/no change. The mixed categorization was also given to proteins where one peptide promoted activity and the other impeded it. The greatest change observed for either peptide determined the significance represented. The pathway was assembled with the assistance of KEGG Pathway Database. A) Colorimetric representation of changes in activity are based upon altered phosphorylation levels only. B) Alterations in phosphorylation within the PI3K/AKT signaling pathway. C) Alterations in phosphorylation within the MAPK pathway.

## 5.4 Discussion

To test the hypothesis that enhancement of APC activity reverses MDR behaviour through the restoration of two defective APC-dependent cellular mechanisms (stress response networks and mitotic regulation), we performed several complementary studies. Focusing first on the influence of APC activity on stress pathways, we were initially guided by our previous yeast studies that linked them together (Malo *et al.*, 2016; Postnikoff *et al.*, 2012). In these studies, we were able to demonstrate a direct pathway connecting elevated APC activity to enhanced stress responses through Fkh1 and Fkh2 signaling in yeast (orthologs to mammalian FOXO proteins). To expand this to the human model system, and to gain a more global perspective of alterations to the activity of the stress response networks in response to APC activity, a kinome array was performed which revealed altered phosphorylation levels over a broad series of interrelated stress and proliferation pathway proteins.

Significant changes to the MAPK and PI3K signaling pathways invoked by APC activation upon peptide expression highlight the significant role of APC in regulating stress response and proliferative pathways (**Figure 5.9**). Within each signaling pathway there are many proteins of known importance, and we noted that the changes influenced by APC activation/peptide expression were wide and impacted various signaling pathways. The kinome array therefore revealed specific proteins whose activities were altered in response to APC activation. As discussed below, we observed numerous phospho-modifications consistent with downstream increases/promotion of stress response signalling, with a simultaneous inhibition of proliferation pathway signalling. (**Figure 5.9**). The kinome array also identified a series of pro-oncogenic pathways that experienced decreased activity upon augmented APC activity. This indicates that enhanced APC activity serves to promote anti-tumorigenic behaviour through cellular networks, which may play a role in cancer responsiveness to chemotherapy.

| Protein | Peptide | Altered Abundance (% control) | p-Value | Figure |
|---|---|---|---|---|
| HURP | C13.3 | 44.4 | 0.0316 | 4.4 |
| | C43.4 | 30.7 | 0.0047 | |
| Cyclin B1 | C13.3 | 73.9 | p<0.0001 | 4.4 |
| | C43.4 | 49.9 | P<0.0001 | |
| CDC20 | C13.3 | 49.1 | 0.0702 | 4.4 |
| | C43.4 | 58.3 | 0.2372 | |
| Cyclin A2 | C13.3 | 70.6 | 0.3625 | 5.2 |
| | C43.4 | 105 | 0.4426 | |
| NEK2A | C13.3 | 67.3 | 0.1208 | 5.2 |
| | C43.4 | 84.7 | 0.4964 | |
| p-PPP1CA T320 | C13.3 | 65.4 | 0.0028 | 5.3 |
| | C43.4 | 68.6 | 0.0583 | |
| p-AKT S473 | C13.3 | 226 | 0.1535 | 5.3 |
| | C43.4 | 83.9 | 0.0749 | |
| p-FOXO3A S253 | C13.3 | 58.8 | 0.0031 | 5.3 |
| | C43.4 | 54.6 | 0.0028 | |
| p-FOXO3A (energy stress) | C13.3 | 39.9 | 0.0295 | 5.4 |
| | C43.4 | 43.8 | 0.0063 | |
| BCRP | C13.3 | 117 | 0.1108 | 5.5 |
| | C43.4 | 76.1 | 0.1824 | |
| MDR-1 | C13.3 | 149 | 0.2358 | 5.5 |
| | C43.4 | 82.0 | 0.1791 | |
| cPARP | C13.3 | 161 | 0.1062 | 5.6 |
| | C43.4 | 210 | 0.1591 | |
| γH2A.X | C13.3 | 110 | 0.2396 | 5.8 |
| | C43.4 | 172 | 0.0532 | |

**Table 5.1. Altered protein abundances resulting from peptide expression.** A summary of the observed alterations on protein accumulation resulting from C13.3 and C43.4 expression.

Through the observed elevated degradation of the APC substrates Cyclin A2, Cyclin B2, and NEK2A, we were able to establish a pathway linking enhanced APC activity with elevated stress responses. While both peptides provoked significant degradation of Cyclin B1 (thereby inhibiting CDK1, **Figure 4.4 A, D**), they demonstrated preference in promoting the degradation of Cyclin A2 and NEK2A; C43.4 supported greater degradation of Cyclin A2 (thereby inhibiting CDK2), whereas C13.3 induced a greater degree of NEK2A degradation (**Figure 5.2**). This altered substrate preference likely presented itself in multiple uncharacterized proteins that we did not

select for further analysis, and likely accounts for many of the discrepancies observed between C13.3 and C43.4 induced behaviour. This may be a more accurate model of describing the augmented APC activity than the broad stroke "greater elevation in activity" when discussing differences observed between peptide expressing cell lines (as discussed in section 4.4).

Based on the observed increase in PPP1 activity induced by peptide expression, we would expect AKT1 signaling to be impaired (**Figure 5.3 A, B, C**). However, this elevated PPP1 activity did not result in a significant downstream dephosphorylation of AKT1, as we predicted, and as has been previously described (Thayyullathil *et al.,* 2011). Based on the phosphorylation of AKT residue S473, C43.4 provided a limited inhibition of AKT1, while C13.3 appears to have elevated activity. There are other key activating sites on AKT1 including Threonine308 (T308) and Threonine450 (T450) that may have been affected, but were not explored through western blotting (Sarbassov *et al.,* 2005; Xiao *et al.,* 2010). The kinome array contained three conserved residues between AKT isoforms AKT1 and AKT3 Threonine305, Threonine447, and Serine472 (conserved as T308, T450, and S473 respectively on AKT 1 respectively; Vasudevan and Garraway, 2010) which indicated an impairment to AKT3 activity (**Figure 5.9 B and Appendix A.1**).

Despite the failure to conclusively demonstrate impaired AKT1 activity based on activating site phosphorylation, multiple observations were made that would indicate an overall impairment of AKT1 activity. A key component of AKT's oncogenic signaling is through FOXO3A inhibition which results in increased proliferation and cell survival (Brunet 1999). This mechanism has been explored for therapeutic efficacy using an approach by pharmacologically inhibiting AKT activity in order to promote FOXO3A transcription, and has been described in not only breast (Dey *et al.,* 2015), but also in pancreatic (Roy *et al.,* 2010), leukemia (Thayyullathil *et al.,* 2011) and colon cancers (Shoeb *et al.,* 2013) demonstrating therapeutic potential for this approach. Despite the lack of significant AKT alterations, we observed with both C13.3 and C43.4 a marked reduction in inhibitory phosphorylation performed by AKT1 (**Figure 5.4 A, C**).

Another consequence of heightened AKT signaling is an induction of MDR behaviour through an increase in the abundance of plasma membrane drug efflux pumps that enable malignant cells to avoid lethal doses of chemotherapy (Goler-Baron *et al.,* 2008; Zhou *et al.,* 2013). Knowing this, we investigated the impact of peptide expression and APC activation of ABC transporter abundance. The observations in C43.4 cells indicates a potential reduction in both ABC transporter proteins tested, MDR-1 and BCRP, although the results were not sufficient to be considered significant (**Figure 5.5**). This observation supports the idea of C43.4 inhibiting AKT signalling, as AKT activity promotes ABC transporter accumulation (Goler-Baron *et al.,* 2008, Zhou *et al.,* 2013). The restoration of chemosensitivity occurred most potently with the C43.4 peptide, and given that apoptotic signaling was equal between the two peptides under non-cytotoxic condition, but elevated to a greater extent in C43.4 upon DOX exposure (**Figure 5.6**), the reduced presence of ABC transport proteins possibly contributed to this phenomenon.

The impact of increasing APC activity through peptide expression was also observed by the elevated responsiveness of 231 cells to metabolic stresses, namely a nutrient/energy stress arising from growth in limited glucose media; the increases in FOXO3A activity induced by APC

activity was augmented by nutrient and energy stress. Specifically, FOXO3A was more active in low glucose, as compared to high glucose, media (**Figure 5.3 A, D and Figure 5.4**). This adaptation of stress pathway activity indicates an elevated capability of the cells to respond to not only the cytotoxic stress of DNA damaging agents such as the chemotherapy DOX, but also to retain robust responses to the stresses of energy deprivation. This is relevant to one of the hallmarks of cancer development, the Warburg Effect where cancer cells ramp up a modified energy metabolism shifted towards glycolysis and lactic acid synthesis, and away from oxidative phosphorylation (Hanahan and Weinberg, 2011; Vander Heiden *et al.,* 2017). This energy-depleted state should induce AMPK activation and signalling (anti-oncogenic), and a simultaneous suppression of nutrient signalling through mTORC activity (pro-oncogenic) and AKT, to prevent proliferation and induce stress responses. This shift in metabolism is thought to be reinforced by the hypoxic environment of tumors (DeBardainis *et al.,* 2008). This is relevant when considering the interplay between proliferative and stress response circuitry response to stressful stimuli as a therapeutic route, and the impact APC activity may therefore have. For instance, caloric restriction and caloric restriction mimetics (rapamycin and metformin) have demonstrated themselves to be promising adjunct therapies to improve clinical cancer outcomes (Meynet and Ricci, 2014).

Expression of two APC-activating peptides appears to shift signalling pathways in our MDR cell line in a way that apoptosis is restored and chemosensitivity is enhanced. In 2001, it was demonstrated that restoring apoptotic pathways, by inducing Caspase 3 overexpression, was an effective method to overcome/reverse MDR behaviour (Friedrich *at al.,* 2001). Caspase activity, central to the apoptotic pathways, is commonly found to be impaired in human tumors as a means of avoiding apoptosis (Olsson and Zhivotovsky, 2011). Anti-stress responses, particularly AKT-dependent activities, are capable of both directly impairing caspase activity through direct inhibition of caspase proteins, as well as altering the regulation of BCL-2 family proteins to inhibit apoptosis (Datta *et al.,* 1997; Gardai *et al.,* 2004; Fernald and Kurokawa, 2013). C43.4, which provoked the greater elevation in apoptosis as measured by PARP cleavage (**Figure 5.6 A-D**), also induced the greater elevation in sensitivity to chemotherapy (**Figure 4.5**) as compared to peptide C13.3. This supports our conclusion that the elevated cell death observed upon peptide expression is likely a result of enhanced apoptotic pathways. The elevated apoptotic rates observed in response to DOX exposure was not proportional to the FOXO3A elevation, therefore it is likely that multiple mechanisms are simultaneously promoting sensitivity to DOX, including MDR transporter abundances, as mentioned previously. Another potential mechanism that may be induced by peptide expression, but not explored here, would be the presence of necrosis induction in response to chemotherapy. Our data therefore supports our secondary hypothesis, that enhancing APC activity reverses MDR behaviour through restoration of defective cellular mechanisms.

Mitotic errors and DNA damage accumulation were the second key APC function investigated in our MDR model, in order to determine the impact of APC activity on cancer behavior. We observed that for both peptides, elevating APC activity resulted in a significantly increased rate of dysregulation in mitosis. We found clear evidence that enhancing APC activity in 231 cells dramatically increased the frequency of mitotic catastrophes even in the absence of

cytotoxic chemotherapy (**Figure 5.6**). This contradicts our hypothesis stating that enhancing APC activity will restore cellular processes to normal, and instead highlighted the possibility of an unknown and alternative pathway whereby normalized APC activity can result in enhanced 'endogenous' cancer cell death rates, and ultimately improve treatment responses to therapy.

A clue to the underlying cellular mechanism causing this enhanced mitotic catastrophe comes from the reported *in vitro* effects of two commercial APC activators, Mad2 inhibitors (M2I-1) and TTK kinase inhibitors (TTKi). It remains to be determined how the peptides may actually influence APC activity, but the end result appears to be mechanistically similar to that observed with these commercial activators. Both M2I-1 and TTKi treatments have been demonstrated *in vitro* to promote a dysregulated mitosis that induces catastrophic genomic damage and results in malignant cell death as monotherapy, under high doses (Li 2019, Maia 2018, Wenger 2016). However, the *in vitro* combination of M2I-1 and TTKi have demonstrated synergistic anti-tumorigenic affects with chemotherapy agents (Li 2019, Maia 2018, Wenger 2016), again producing a result similar to our *in vitro* studies using peptides C43.4 and C13.3. and DOX (**Figure 4.5**).

A mechanistic explanation for the altered MDR behaviour upon APC activation should consider the possibility that activation of cell cycle checkpoints (specifically the SAC in this instance) may slow down the cell cycle and replication, enabling the partial repair of defects occurred during mitosis. It has been reported that cancer cells with impaired APC activity experience reduced chromosomal instability (Sansregret *et al.,* 2017). This reduced chromosomal instability and may function similarly to that observed with restoration of BRCA1 and BRCA2 activity. Here, there is partial repair of the genome, thereby preventing the cell death induced by chemotherapy (Husain *et al.,* 1998; Sakai *et al.,* 2008). By enhancing APC activity and inducing mitotic catastrophes, the burden of elevated chromosomal instability may act to promote cell death (Birkbak *et al.,* 2011). This may occur through both $APC^{CDC20}$ and $APC^{CDH1}$, as despite CDC20 experiencing enhanced degradation upon peptide expression (**Figure 4.4 A, C**), the enhanced degradation of NEK2A also indicates that $APC^{CDC20}$ is also specifically elevated, as NEK2A degradation is exclusively driven by CDC20. As mentioned in Section 4.4, it appears that CDH1-dependent APC activity is clearly elevated, as noted by the degradation of CDC20 (by both peptides), which is exclusively driven by CDH1. Therefore, it is also possible that CDH1 is inducing the mitotic dysregulation through promoting mitotic slippage (Nagai *et al.,* 2014; Toda *et al.,* 2012; Park *et al.,* 2018).

The peptides also were noted to modestly increase the number of cells within a population residing in mitosis, and there are multiple possible interpretations for this (**Figure 5.6 B**). The first could be that enhancing APC activity encourages cells to enter mitosis, possibly in a dysregulated manner. An accelerated cell cycle implies bypassing cell cycle checkpoints, potentially overcoming MDR behaviour induced through checkpoint activation (Harrington *et al.,* 1994; Ferrao *et al.,* 2012; Morgan *et al.,* 2010). The second interpretation is that APC activity slows the process of mitosis, meaning more cells exist in mitosis at any given time. This is frequently seen in various mutant *S. cerevisiae* strains, where the proportion of cells rises greatly to reside

preferentially in one stage of the cell cycle. 231 cells expressing peptides exhibited a modest increase in mitotic figures per population would indicate a more regulated mitosis is occurring, as mitotic checkpoint activation is arresting mitosis. However, this is contradictory to published observations using M2I-1 to activate the APC *in vitro*. Here, impaired SAC activity reduced the time in mitosis (Kastl 2015). One consequence of an excessively long mitotic arrest is accrual of DNA damage and potentially mitotic catastrophe (Bonaiuti *et al.,* 2018, Dalton *et al.,* 2007; Quignon *et al.,* 2007). The former interpretation of promoting cell cycle progression better fits the current data, however further investigation is essential to clarify/elucidate the underlying mechanisms resulting in our observed impacts on mitosis upon APC activation in MDR cells.

It is also important to discuss the increased rates of DNA damage upon APC activation with peptides, in cells simultaneously exhibiting mitotic dysregulation (**Figure 5.7**). Again, this was counterintuitive to our hypothesis, where we posited that DNA damage would in fact be decreased due to enhanced repair rates. We considered how this observation might relate to the enhanced chemosensitivity found upon peptide expression. Clearly, the induction of (catastrophic) DNA damage through pharmacological agents has long been a staple of cancer chemotherapy, including the general drug classes of alkylating (cisplatin)s and anthracycline (DOX) agents, for example. Therefore, it is possible that the elevated DNA damage induced through enhanced APC activity pushes the cancer population closer to irreparable genomic damage, and makes the cells more susceptible to DNA damaging agents (Birkbak *et al.,* 2011). C13.3 however was unable to induce an elevation in γH2A.X accumulation. The cause of this phenomenon is unknown, however one potential explanation is that C13.3 induces more catastrophic accumulation of DNA damage than C43.4 and is more likely to induce cell death through necrosis (a mechanism we have not characterized) even without the addition of the chemotherapeutic agent.

Several clear differences in our endpoints were found between the C13.3. and C43.4 peptides, however this was not further examined in our experiments. There are many explanations for the different outcomes between them, including the fact that the peptides were initially identified in yeast through binding to different APC subunits (**Table 4.1**). In the yeast 2-hybrid library, peptide C13.3 bound to the APC5 subunit (important for APC structural integrity and E3 activity) and C43.4 bound the APC10 subunit (essential for substrate recognition). Importantly, we have not yet confirmed that either peptide expressed in 231 cells is directly binding the human APC, but could also have unique off-target interactions that may influence APC activity and cellular pathways to reverse MDR behaviors. This may drive differential activation of cellular pathways to enhance unique cellular pathway activity depending on the peptide used. Overall, the behavioural changes demonstrated by the elevated stress response pathways, apoptosis, MDR reversal, and kinome array indicate that the APC overall acts as a tumor suppressor protein. This corresponds with the known functions of the APC in healthy cells, including protecting the genome and inducing stress responses, apoptosis, and cell cycle arrest.

# Chapter 6. Conclusion

This thesis investigated two hypotheses. Our primary hypothesis was supported by our investigations and demonstrated that enhancement of APC activity results in reversal of MDR behaviour in cancer and restoration of chemosensitivity. Our investigation utilized novel peptides identified in a yeast 2-hybrid library as being capable of directly binding APC subunits and enhancing yeast APC activity. We adapted the peptides to be endogenously expressed in the innately MDR cancer line MDA-MB-231. This resulted in two peptides, C13.3 and C43.4, being confirmed as being capable of enhancing APC activity. This elevated APC activity coincided with a significantly elevated sensitivity to the chemotherapeutic DOX. Our secondary hypothesis addressed the possibility that the targeted enhancement of APC activity would correct defects that promote MDR behaviour, including normalised mitotic progression, and decreases in DNA damage accumulation. In support of our secondary hypothesis, we did reveal evidence that stress responses and apoptosis was increased upon peptide expression and APC activation in the MDR cell model. We were able to construct a cellular pathway connecting the observed degradation of multiple APC protein substrates relevant to the stress response networks and altered signalling within the networks themselves. Notably, we detected marked changes in the phosphorylation of PPP1. This is indicative of elevated PPP1 activity, promoting the dephosphorylation of protein targets to repress cell proliferation, and enhance apoptosis and repair.

Our results when investigating the next step in the pathway, AKT1 were mixed. We noted a mild decrease in AKT activity with C43.4, and an elevation with C13.3. However, we observed multiple alterations to cellular behaviour indicative of a net impairment of AKT activity with both peptides. The most important observation was the promotion FOXO3A activity, indicating the induction of stress response pathways. We did observe that peptide expression markedly reduced the inhibitory phosphorylation of FOXO3A performed by AKT1. This occurred under both rich and nutrient/energy limiting conditions, with a subsequent elevation in several apoptotic markers both endogenously and also when exposed to the chemotherapy agent DOX. Together, this suggest that by enhancing APC activity, we are restoring defective apoptotic pathways. As revealed by the kinome array, enhancing APC activity also broadly as induced extensive alterations to protein phosphorylation of multiple stress response proteins. In both the MAPK and PI3K pathways these changes broadly indicate the activation of stress responses while simultaneously suppressing proliferation.

Contrary to our secondary hypothesis, the enhancement of APC activity resulted in increased rates of mitotic dysregulation. This likely acted to enhance the sensitivity of the MDR cancer cells to the therapeutic and cytotoxic effects of chemotherapy, as a dysregulated mitosis is one of the key mechanisms through which cancer cells accrue DNA damage. This elevated DNA damage may synergize with DOX (which functions through inducing DNA damage), to induce cell death. Therefore, the activity of the APC is intrinsically tied to the presence of MDR behaviour.

# Chapter 7. Future Directions

This adaptation of APC-activating peptides for human expression and investigation into MDR behavior was limited in scope, as it was performed exclusively in MDA-MB-231 cells, and it is therefore difficult to draw conclusions regarding the applicability of this research to other cancer types. Therefore, replication of these studies should be performed in a second complementary *in vitro* model. One excellent candidate would be to use paired cancer cell lines that are matched except for chemosensitivity. Our lab is well positioned to generate acquired chemoresistance cell lines to study MDR behaviour, rather than innately MDR observed in the 231 cells, as we have previously generated such paired lines in human leukemia, lymphoma, and estrogen receptor positive breast caner cells. This would not only verify the impact of elevating APC activity in a secondary cell line, but may yield data on the mechanisms at play in cells undergoing an MDR transformation. The ability to induce a MDR phenotype is also a unique opportunity to determine if APC activation can in fact prevent drug resistance from developing. This is clinically relevant when considering patients who have excellent initial responses to their chemotherapy, but return years later with MDR. It may be that a role for chronic low-level APC stimulation could be clinically significant in preventing these untreatable recurrences. The data presented were also exclusively *in vitro*, and are therefore missing crucial data on the impact that these peptides have in an *in vivo* model. To study an *in vivo* model, peptide expressing 231 cells would be implanted into immunocompromised mice to develop solid tumors. These tumors could then be evaluated for several measures of cancer behaviour. This would include the impact on tumor growth, and responsiveness to chemotherapy. These *in vitro* studies are currently underway by Gabrielle Mercier and Dr. Gerald Davies.

Currently we have little understanding of how the peptides of interest function to drive APC activity. The most important aspect of peptide behaviour to demonstrate would be whether they are biologically functional due to direct APC interactions, indirect effects due to unique off-target associations, or a combination of both. Direct associations between the peptides and the human APC complex could be addressed by several means. Co-immunoprecipitation assays would confirm peptide binding to the APC protein complex, as co-immunoprecipitation would pulldown the entire E3 with all bound accessory proteins; however, the information potentially obtained about peptide binding is limited due to the entire complex being retrieved when a single subunit is targeted so that the interacting subunit would not be necessarily identified. Another binding assay that has the advantage of highlighting protein proximity to the peptide of interest would be BioID, where the peptides are fused to a mutated biotin ligase that promiscuously tags nearby proteins. These tagged proteins can then be purified through biotin affinity purification, and then identified through mass spectrophotometry. BioID would provide an interaction map of the peptide, as well as potentially identify nearby proteins that associate with the APC, therefore also identifying potential off-target interactions that are biologically relevant. Proteins located closer to peptides would produce a stronger signal in the screen, thereby helping to identify a more specific binding

location. Based on known regulatory measures, this may identify the mechanisms by which the peptides enhance APC activity.

Further investigation of impact of these peptides on cell cycle regulation is warranted. The elevated mitotic catastrophes indicate that there is a loss of mitotic regulation that occurs from enhanced APC activity; however, the underlying mechanisms are not understood. This would involve several experiments. First, observation of the cell cycle following synchronization. This will indicate if the peptides alter the cell cycle, with respect to the timing of the different sections of interphase, and the impact of enhanced APC activity on cell cycle checkpoints. Most importantly, this will establish the impact of APC activation on mitotic timing. Are regulatory mechanisms being activated or impaired by enhanced APC activity? These experiments would be repeated from multiple points of the cell cycle to gain a thorough picture of cell cycle regulation. A second experiment will focus specifically on mitotic regulation. We could examine the impact of APC activation on the induction of mitotic slippage by using chemical inhibitors of mitotic progression. The question being asked is do peptide expressing cells experience exaggerated mitotic arrest, or do they experience slippage at an elevated rate when compared to control? This would elaborate on the mechanisms that drive mitotic regulation, and verify our current data demonstrating APC activity promoting mitotic dysregulation.

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# Appendix A

## A.1 Alterations to Pathway Activity Identified in the Kinome Array

**MAPK signaling Pathway:** The mitogen-activated protein kinase (MAPK) pathway contains many oncogenes commonly found to be overexpressed, or experience gain-of function mutations in cancer. Activation of the APC generated a downregulation of the pathway. Two principle initiators of the MAPK pathway are the receptor tyrosine kinases (RTKs) EGFR and Kit. Active EGFR and Kit phosphorylate SHC1, a linker protein which forms protein complexes to transduce the signal from RTKs to induce the MAPK signaling pathways. A key function of SHC1 is the binding of the guanine nucleotide exchange factor (GEF) SOS1 to facilitate its activation by localizing it to the RTKs. SOS1 would then promote the activity of the Ras family of proteins including Rac1 and HRas, through exchanging bound GDP (inactive state) for GTP (active state). Downstream of Rac1 and HRas are BRAF and RAF1. BRAF and RAF1 are directly phosphorylated by HRas. An alternative method of activating MAP3K proteins is through the PAK proteins, whose activity can be induced by either JAK/STAT signaling or Rac1. Both RAF1 and BRAF phosphorylate MEK1/2 to promote their activity, which in turn activates ERK2.

EGFR, Kit, and SHC1 experienced a decrease in activity, impairing pathway activation. By inhibiting SOS1, Rac1 and HRas are inhibited through elevated GDP binding (although this is not examined by the kinome array. Rac1 and HRAS also experienced a loss of activity through altered phosphorylation levels. BRAF found to have a loss of activity while RAF1 had elevated activity. The elevation in RAF1 activity is likely through activation of the JAK/STAT pathway. The gain in JAK signalling compensates for the loss of Rac1 and HRas activity through driving PAK1 activity. This is demonstrated in the loss of phosphorylation of Rac1 targeted residues and the gain in phosphorylation JAK1 targeted residues. PAK1 then phosphorylates RAF1, driving its activity and activating the MEK1. MEK2 was not significantly altered to either enhance, or inhibit activity. ERK2 was found to have a decrease in activity however, despite the elevation in MEK1 activity. This is likely from elevated DUSP1 activity which dephosphorylates ERK family proteins to inhibit their activity. Examination of EKR2 substrates verified the loss of activity ERK2, as FOS, JUN, and MSK1 experienced a loss of activity. Ribosomal protein S6 kinase (RPS6) alpha-3 (RPS6KA3) activity was slightly enhanced, potentially acting as a stop signal for MAPK signaling by inhibiting SOS1.

**PI3K:** The PI3K pathway had conflicting signaling pathways. Insulin receptor (IRS) signaling was elevated, therefore driving activity of the PI3K family. PI3K isoforms B and D were found to have upregulated activity, but isoform G experienced decreased activity. Simultaneously PTEN was upregulated. The net result of these conflicting pathways is decreased signaling downstream of $PIP_3$ as indicated by the critical loss of the critical activating autophosphorylation of 3-phosphoinositide-dependent protein kinase-1 (PDK1), which is phosphorylated upon interaction with $PIP_3$. There were both mild elevations to mTOR activyt, as well as one significant inhibitory event, resulting in the net impact on mTOR activity being mixed. The loss of PDK1 activity corresponded with a reduction in the activity of PDK1 substrates within the AKT and PKC protein families. The effects of the altered PKC signaling is demonstrated in the moderate downregulation of Protein Kinase D1 (PRKD1) activity which acts as intermediary for may of the PKC family proteins in promoting their pathways.

**AKT:** AKT exists as 3 isoforms, AKT1, 2, and 3. Activating proteins of the AKT family, PKD1, Heat Shock Protein 90 Beta Family Member 1 (HSP90B1), mTOR, and PKCA were all found to be downregulated in activity. Overall effect of this altered phosphorylation pattern indicates an overall decrease in activity of AKT isoforms. AKT 3 was the best characterized protein in the array and was found to be downregulated through loss of multiple activating phosphorylation sites. Only one AKT1 site (T450) was present in the final data set, and demonstrated moderate dephosphorylation. Due to conservation between isoforms, dephosphorylation of AKT 3 is likely to indicate dephosphorylation of AKT1. Examination of the AKT phosphorylation sites on Raf-1, Glycogen Synthase Kinase 3 Alpha (GSK3α), SMAD3 revealed a loss of phosphorylation at sites performed by AKT proteins, indicating a reduction in AKT activity. CDK1 and CDK2, while not directly phosphorylated by AKT, experience activity promotion through upstream AKT activity. The loss of AKT signaling correlated with a loss in CDK1 and CDK2 activity.

**MTORC1:** Given the mixed alteration to mTOR, the distinct complexes may experience separate alterations to activity. Regulatory-associated protein of TOR 1 (RAPTOR) experienced a decrease in activation, indicating that MTORC1 activity is likely impaired. Two substrates of MTORC1 experienced altered phosphorylation indicating MTORC1 inhibition, EIF4BP1 (eukaryotic initiation factor 4 [EIF4] binding protein) and RPS6KB1. EIF4BP1 is a negative allosteric inhibitor of EIF4. By preventing EIF4 activity, elevated EIF4BP1 is reducing global protein synthesis as EIF4 binds mRNA to induce protein translation. RPS6KB1 induces multiple events to promote proliferation prevent apoptosis and experienced a decrease in activity.

**TGFβ:** Transforming growth factor beta (TGFβ) receptor 1 (TGFBR1) experienced a loss of activating phosphorylation. Despite this, SMAD 1 and 2, substrates of TGFBR1 kinase activity experienced an elevation in phosphorylation to promote activity. Bone morphogenetic protein receptors (BMPRs) were not included in the final data set, however an upregulation of their activity

would promote SMAD2 phosphorylation (but not SMAD1), and therefore activity. SMAD 3 experienced a loss of inhibitory phosphorylation from AKT1 and multiple CDKs, notable from the observed loss of CDK2. The SMAD proteins would then be free to dimerize in the nucleus to transcribe proteins in an anti-tumorigenic manner.

**NF κ B:** Nuclear factor kappa-light-chain-enhancer of activated B cells **(**$NF_κB$) signalling is initiated through activity of inhibitor of nuclear factor kappa-B kinase (IKK) proteins. The signaling was mixed as IKKα experienced an elevation in activity, while IKKε experienced a decrease in activity. There are multiple proteins not characterized in the kinome array that may explain the upregulation of IKKα activity (the IKKα sites phosphorylated by AKT1 were not characterized by the array). IKKε activity is primarily driven by EGFR, and the reduction in EGFR activity explains this loss of activity. IKK proteins induce $NF_κB$ signalling through phosphorylation of $NF_κB$ 1 and NFκB Inhibitor alpha (NFKBIA; an inhibitory chaperone of $NF_κB$). The phosphorylation of NFκB 1 and 2 promotes their activity, and the phosphorylation of NFKBIA promotes its degradation. NFKBIA experienced a loss of these phosphorylation events. Phosphorylation of $NF_κB$ 1 indicated an increase in activity, however the impact on transcriptional activity is not certain as NFKBIA is still abundant and may be downregulating NFKIBA activity through allosteric regulation. $NF_κB2$ experienced a direct decrease in activity through phosphorylation. Due to the complicated transcriptional networking involving $NF_κB$ 1 dimerization with multiple transcriptional factors not included in the array, a solid conclusion on the overall outcome of the altered $NF_κB$ activity would only be speculative. The main conclusion to be reached is that $NF_κB$ 2 signaling be downregulated upon APC activation.

**JAK/STAT:** The JAK/ STAT signaling pathways are promoted by an elevated abundance of Interferon alpha/beta receptor 1 (INFAR1), as indicated by a decrease in the phosphorylation site that promotes degradation. INFAR1 is then capable of promoting the activity of JAK proteins. Suppressor of cytokine signaling 3 (SOCS3) allosterically regulates JAK activity by binding phosphorylated JAK proteins (in a negative-feedback pathway), and exhibited elevated activity. The upregulation of SOCS3 activity does not appear to be sufficient to completely suppress JAK signaling, as while STAT 1 and 3 both experience a decrease in activating phosphorylation induced by JAKs, as mentioned previously, PAK 1 experienced a mild increase in activating phosphorylation from JAK1.

**Apoptosis:** 14-3-3ζ binds and inhibits activity of Bcl2-associated agonist of cell death (BAD), which promotes apoptosis through inhibition of the anti-apoptotic proteins B-cell lymphoma 2 (BCL-2) and BCL extra large (Bcl-xL). The elevated phosphorylation of 14-3-3ζ results in its dissociation from BAD, to permit apoptosis. The activity of MAPKPK2 and the kinase function of Bcr and were both elevated to perform these inhibitory events. This would permit BAD activity to permit initiation of mitotic pathways. Caspase 3 activity was also elevated through phosphorylation events. This is in part due to the reduction in P38A activity, which inhibits

Caspase 3 activity. Many of the above discussed pathways may either promote or impede apoptosis. The general trend of the alterations to these pathways resulted in a promotion of apoptosis.

## A.2 Kinome Data for C13.3

| ID | Accession | Sequence | FC | P up | P down | Beta up | Beta down | Negative predictive value up | Negative predictive value down |
|---|---|---|---|---|---|---|---|---|---|
| P42345 | S2449 | RSRTRTDSYSAGQSV | 1.28010 | 0.00997 | 0.99003 | 0.11432 | 0.99992 | 0.07501 | 0.65613 |
| P42345 | S2482 | TVPESIHSFIGDGLV | 1.26664 | 0.05801 | 0.94199 | 0.14155 | 0.99986 | 0.09288 | 0.65610 |
| P42345 | S1261 | PMKKLHVSTINLQKA | -1.59115 | 0.99926 | 0.00074 | 1.00000 | 0.00008 | 0.65619 | 0.00005 |
| Q8N122 | S863 | LTQSAPASPTNKGIH | -1.40957 | 0.99299 | 0.00701 | 1.00000 | 0.00787 | 0.65619 | 0.00517 |
| Q13131 | S486 | DEITEAKSGTATPQR | 1.20467 | 0.06319 | 0.93681 | 0.25727 | 0.99935 | 0.16882 | 0.65576 |
| P35568 | S1095 | GCRRRHSSETFSSTP | -1.35057 | 0.98164 | 0.01836 | 1.00000 | 0.00488 | 0.65619 | 0.00320 |
| P48736 | S582 | LWHFRYESLKDPKAY | -1.87502 | 0.99964 | 0.00036 | 1.00000 | 0.00091 | 0.65619 | 0.00059 |
| P42338 | Y505 | ENKKQPYYYPPFDKI | 1.27398 | 0.01073 | 0.98927 | 0.07851 | 0.99997 | 0.05152 | 0.65616 |
| P42338 | Y963 | RVPFILTYDFIHVIQ | -1.24520 | 0.90407 | 0.09593 | 0.99943 | 0.24472 | 0.65582 | 0.16058 |
| P42338 | S1071 | KVNWMAHTVRKDYRS | -1.98467 | 0.99692 | 0.00308 | 1.00000 | 0.00854 | 0.65619 | 0.00560 |
| O00329 | S1021 | KVNWLAHNVSKDNRQ | -1.90299 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| O00329 | Y524 | RRGSGELYEHEKDLV | -1.22603 | 0.96069 | 0.03931 | 0.99998 | 0.06233 | 0.65617 | 0.04090 |
| P00533 | Y1172 | PAAENAEYLRAAPAG | -1.88178 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| P00533 | Y1067 | TFLPVPEYVNQSVPK | 1.34304 | 0.00568 | 0.99432 | 0.00223 | 1.00000 | 0.00146 | 0.65619 |
| P00533 | Y846 | LGAEEKEYHAEGGKV | 1.20403 | 0.09046 | 0.90954 | 0.28746 | 0.99911 | 0.18863 | 0.65560 |
| Q15831 | S309 | SASSKIRRLSACKQQ | -1.32964 | 0.98186 | 0.01814 | 0.99999 | 0.04213 | 0.65618 | 0.02765 |
| P60484 | S346 | EPDHYRYSDTTDSDP | -1.63533 | 0.99341 | 0.00659 | 1.00000 | 0.00085 | 0.65619 | 0.00055 |
| P60484 | Y104 | TGVMICAYLLHRGKF | -1.87008 | 0.99997 | 0.00003 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| O15530 | Y374 | SEDDEDCYGNYDNLL | 1.42421 | 0.00138 | 0.99862 | 0.00015 | 1.00000 | 0.00010 | 0.65619 |
| P17252 | T401 | MDGVTTRTFCGTPDY | 1.28839 | 0.01934 | 0.98066 | 0.09675 | 0.99994 | 0.06349 | 0.65615 |
| P17252 | T542 | TRGQPVLTPPDQLVI | -1.82660 | 0.99977 | 0.00023 | 1.00000 | 0.00029 | 0.65619 | 0.00019 |
| Q02156 | S368 | NNIRKALSFDNRGEE | 1.51688 | 0.00003 | 0.99997 | 0.00001 | 1.00000 | 0.00001 | 0.65619 |
| Q02156 | S729 | QEEFKGFSYFGEDLM | -1.22567 | 0.94313 | 0.05687 | 0.99967 | 0.20012 | 0.65597 | 0.13131 |
| Q02156 | T566 | LNGVTTTTFCGTPDY | -1.48242 | 0.99553 | 0.00447 | 1.00000 | 0.00931 | 0.65619 | 0.00611 |
| Q04759 | S695 | QNMFRNFSFMNPGME | -1.65764 | 0.99889 | 0.00111 | 1.00000 | 0.00027 | 0.65619 | 0.00018 |
| Q04759 | T538 | LGDARTNTFCGTPDY | -1.85084 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| Q04759 | Y90 | SETTVELYSLAERCR | -2.98488 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| Q9Y243 | S472 | RPHFPQFSYSASGRE | -1.37883 | 0.95837 | 0.04163 | 0.99997 | 0.07125 | 0.65617 | 0.04676 |
| Q9Y243 | S120 | EEERMNCSPTSQIDN | -1.25256 | 0.98009 | 0.01991 | 0.99995 | 0.09195 | 0.65615 | 0.06034 |
| Q9Y243 | T305 | TDAATMKTFCGTPEY | -1.79159 | 0.99171 | 0.00829 | 1.00000 | 0.00081 | 0.65619 | 0.00053 |
| P14625 | T165 | ELVKNLGTIAKSGTS | -1.71514 | 0.99937 | 0.00063 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| P14625 | S347 | KPIWQRPSKEVEEDE | -1.71943 | 0.98701 | 0.01299 | 1.00000 | 0.02129 | 0.65619 | 0.01397 |
| P23443 | T252 | HDGTVTHTFCGTIEY | -1.74163 | 0.99239 | 0.00761 | 1.00000 | 0.00111 | 0.65619 | 0.00073 |
| P23443 | S394 | TRQTPVDSPDDSALS | -1.89912 | 0.99996 | 0.00004 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| P23443 | T412 | NQVFLGFTYVAPSVL | 1.21896 | 0.08511 | 0.91489 | 0.25379 | 0.99937 | 0.16653 | 0.65578 |
| Q13541 | S65 | FLMECRNSPVTKTPP | -2.12796 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| Q13541 | T46 | GGTLFSTTPGGTRII | -2.70795 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| Q99558 | T558 | TGDYIPGTETHMAPE | -1.13988 | 0.90370 | 0.09630 | 0.99820 | 0.36432 | 0.65500 | 0.23906 |
| O15111 | S181 | DQGSLCTSFVGTLQY | 1.75679 | 0.00000 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Q14164 S666 | RTQGAQASPPLTAPY | 1.14349 | 0.03148 | 0.96852 | 0.28515 | 0.99913 | 0.18711 | 0.65562 |
| Q14164 S172 | DDDEKFVSVYGTEEY | -1.63766 | 0.99995 | 0.00005 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| P25963 T291 | EDEESYDTESEFTED | -2.63998 | 0.99350 | 0.00650 | 1.00000 | 0.00148 | 0.65619 | 0.00097 |
| P68400 Y182 | DWGLAEFYHPGQEYN | 1.67131 | 0.00760 | 0.99240 | 0.00541 | 1.00000 | 0.00355 | 0.65619 |
| P19838 S337 | FVQLRRKSDLETSEP | 1.64253 | 0.00017 | 0.99983 | 0.00000 | 1.00000 | 0.00000 | 0.65619 |
| P19838 S928 | RDDSICDSGVETSFR | 1.32069 | 0.00217 | 0.99783 | 0.00242 | 1.00000 | 0.00159 | 0.65619 |
| Q00653 S99 | EVDLVTHSDPPRAHA | -1.68338 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| Q00653 S708 | EPLCPLPSPPTSGSD | 1.66975 | 0.00000 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 |
| P06493 T161 | GIPIRVYTHEVVTLW | 1.58714 | 0.00068 | 0.99932 | 0.00020 | 1.00000 | 0.00013 | 0.65619 |
| P24941 Y15 | EKIGEGTYGVVYKAR | 1.22562 | 0.04224 | 0.95776 | 0.21759 | 0.99959 | 0.14278 | 0.65592 |
| P24941 S46 | TETEGVPSTAIREIS | 1.62183 | 0.00019 | 0.99981 | 0.00006 | 1.00000 | 0.00004 | 0.65619 |
| P36897 T189 | LPLLVQRTIARTIVL | -2.08400 | 0.99989 | 0.00011 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| Q15797 S187 | NSHPFPHSPNSSYPN | 1.46335 | 0.00172 | 0.99828 | 0.00452 | 1.00000 | 0.00297 | 0.65619 |
| P84022 T133 | PQSNIPETPPPGYLS | -1.23898 | 0.97638 | 0.02362 | 0.99994 | 0.09740 | 0.65615 | 0.06391 |
| P17612 T201 | RVKGRTWTLCGTPEY | -1.44732 | 0.99835 | 0.00165 | 1.00000 | 0.00564 | 0.65619 | 0.00370 |
| P17612 S339 | EEEEIRVSINEKCGK | -1.88453 | 0.99999 | 0.00001 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| P10721 Y569 | EEINGNNYVYIDPTQ | -1.51782 | 0.99669 | 0.00331 | 0.99999 | 0.03726 | 0.65618 | 0.02445 |
| P29353 Y427 | ELFDDPSYVNVQNLD | -2.04367 | 0.99831 | 0.00169 | 1.00000 | 0.00380 | 0.65619 | 0.00249 |
| P29353 S36 | TPPEELPSPSASSLG | -2.01144 | 0.98321 | 0.01679 | 0.99999 | 0.04955 | 0.65618 | 0.03252 |
| Q07889 T158 | NIRHYEITKQDIKVA | -1.72955 | 0.99987 | 0.00013 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| P01112 T144 | YGIPYIETSAKTRQG | 1.49119 | 0.00001 | 0.99999 | 0.00008 | 1.00000 | 0.00005 | 0.65619 |
| P01112 S183 | DEGGPGCLSCRCLLS | 1.41273 | 0.00745 | 0.99255 | 0.03439 | 0.99999 | 0.02257 | 0.65618 |
| P01112 T35 | FVDEYDPTIEDSYRK | -1.39837 | 0.99702 | 0.00298 | 1.00000 | 0.00931 | 0.65619 | 0.00611 |
| P46940 T1392 | DKMKKSKTIKEDSSL | -1.62356 | 0.99989 | 0.00011 | 1.00000 | 0.00014 | 0.65619 | 0.00009 |
| P46940 Y1459 | LVKLQQTYAALNSKA | -2.54355 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| P46940 S282 | ALFKILQSPALGLRG | 1.52307 | 0.00339 | 0.99661 | 0.00810 | 1.00000 | 0.00531 | 0.65619 |
| P04049 S43 | FGYQRRASDDGKLTD | -1.16740 | 0.91150 | 0.08850 | 0.99926 | 0.26925 | 0.65570 | 0.17668 |
| P04049 S259 | SQRQRSTSTPNVHMV | -1.28330 | 0.95881 | 0.04119 | 0.99995 | 0.09375 | 0.65615 | 0.06152 |
| P17181 S536 | SSQTSQDSGNYSNED | -1.55506 | 0.99969 | 0.00031 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| P17181 Y467 | VFLRCVKYVFFPSSK | 1.22233 | 0.01298 | 0.98702 | 0.09488 | 0.99995 | 0.06226 | 0.65615 |
| P23458 Y1033 | AIETDKEYYTVKDDR | 3.66103 | 0.00000 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 |
| P23458 T686 | HRKSDVLTTPWKFKV | 1.88660 | 0.00400 | 0.99600 | 0.00085 | 1.00000 | 0.00055 | 0.65619 |
| P42224 Y1114 | DGPKGTGYIKTELIS | -1.25932 | 0.93669 | 0.06331 | 0.99971 | 0.19087 | 0.65600 | 0.12525 |
| P40763 Y705 | DPGSAAPYLKTKFIC | -1.48899 | 0.94198 | 0.05802 | 0.99973 | 0.18403 | 0.65601 | 0.12076 |
| Q13153 Y284 | QGASGTVYTAMDVAT | 1.36492 | 0.00030 | 0.99970 | 0.02722 | 1.00000 | 0.01786 | 0.65618 |
| Q13153 S198 | PRPEHTKSVYTRSVI | -1.44372 | 0.92871 | 0.07129 | 0.99975 | 0.17998 | 0.65602 | 0.11810 |
| O96013 S475 | KEVPRRKSLVGTPYW | -1.45048 | 0.99015 | 0.00985 | 0.99995 | 0.09051 | 0.65616 | 0.05939 |
| Q02750 T388 | IGLNQPSTPTHAAGV | -1.44418 | 0.99990 | 0.00010 | 1.00000 | 0.00828 | 0.65619 | 0.00544 |
| Q02750 S222 | VSGQLIDSMANSFVG | 1.51665 | 0.00101 | 0.99899 | 0.01442 | 1.00000 | 0.00946 | 0.65619 |
| Q02750 S298 | RTPGRPLSSYGMDSR | -1.22344 | 0.93124 | 0.06876 | 0.99964 | 0.20653 | 0.65595 | 0.13552 |
| P36507 T398 | TLRLNQPSTPTRTAV | -1.27576 | 0.93278 | 0.06722 | 0.99988 | 0.13271 | 0.65611 | 0.08708 |
| P36507 S306 | RPPGRPISGHGMDSR | -1.29790 | 0.99952 | 0.00048 | 0.99999 | 0.04382 | 0.65618 | 0.02875 |
| P28482 T141 | ADPDHDHTGFLTEYV | 1.36111 | 0.00033 | 0.99967 | 0.00289 | 1.00000 | 0.00190 | 0.65619 |
| P28482 Y187 | HTGFLTEYVATRWYR | -1.56030 | 0.99363 | 0.00637 | 1.00000 | 0.00982 | 0.65619 | 0.00644 |
| P51812 S386 | HQLFRGFSFVAITSD | 1.82719 | 0.00018 | 0.99982 | 0.00000 | 1.00000 | 0.00000 | 0.65619 |
| P51812 S369 | TAKTPKDSPGIPPSA | -1.52814 | 0.97591 | 0.02409 | 1.00000 | 0.02387 | 0.65618 | 0.01566 |
| Q15418 S380 | HQLFRGFSFVATGLM | -1.27279 | 0.97243 | 0.02757 | 0.99995 | 0.09627 | 0.65615 | 0.06317 |
| P05412 Y170 | LHSEPPVYANLSNFN | -1.83530 | 0.99999 | 0.00001 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| P05412 S63 | KNSDLLTSPDVGLLK | 1.36981 | 0.01465 | 0.98535 | 0.02451 | 1.00000 | 0.01609 | 0.65618 |
| Q16539 Y323 | DEPVADPYDQSFESR | -1.43241 | 0.99290 | 0.00710 | 1.00000 | 0.00740 | 0.65619 | 0.00485 |
| P49137 T322 | QSTKVPQTPLHTSRV | 1.61202 | 0.00007 | 0.99993 | 0.00000 | 1.00000 | 0.00000 | 0.65619 |
| P49137 T209 | TSHNSLTTPCYTPYY | 2.03403 | 0.00009 | 0.99991 | 0.00000 | 1.00000 | 0.00000 | 0.65619 |
| P49137 S259 | SNHGLAISPGMKSRI | 1.81316 | 0.00034 | 0.99966 | 0.00026 | 1.00000 | 0.00017 | 0.65619 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| P42574 | S150 | FRGDYCRSLTGKPKL | -1.83092 | 0.99999 | 0.00001 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| P63104 | S58 | VVGARRSSWRVVSSI | 1.61168 | 0.00000 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 |
| P63104 | T110 | LTLWTSDTQGDEAEA | 1.49460 | 0.00347 | 0.99653 | 0.00718 | 1.00000 | 0.00471 | 0.65619 |
| P11274 | S120 | GLPYIDDSPASSPHL | -1.33993 | 0.98753 | 0.01247 | 1.00000 | 0.02008 | 0.65619 | 0.01318 |
| P11274 | Y21 | VSPSPTTYRPFRDKS | 2.96232 | 0.00000 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.65619 |
| Q05655 | S645 | LNEKPRLSYSDKNLI | -1.35829 | 0.98638 | 0.01362 | 0.99999 | 0.04386 | 0.65618 | 0.02878 |
| Q15139 | S642 | ARIIGEKSFRRSVVG | 1.21350 | 0.02857 | 0.97143 | 0.06906 | 0.99997 | 0.04532 | 0.65617 |
| Q15139 | Y367 | NDTGSRYYKEIPLSE | -1.43859 | 0.95949 | 0.04051 | 1.00000 | 0.02800 | 0.65618 | 0.01838 |
| P31749 | T442 | TAQMITITPPDQDDS | -1.45421 | 0.98102 | 0.01898 | 0.99999 | 0.04000 | 0.65618 | 0.02624 |
| P49840 | S39 | GGGGPGGSASGPGGS | -1.36291 | 0.99787 | 0.00213 | 1.00000 | 0.01157 | 0.65619 | 0.00759 |
| P49840 | S39 | GGGGPGGSASGPGGS | -1.36291 | 0.99787 | 0.00213 | 1.00000 | 0.01157 | 0.65619 | 0.00759 |
| P49840 | S21 | SGRARTSSFAEPGGG | -1.40496 | 0.97646 | 0.02354 | 0.99999 | 0.04593 | 0.65618 | 0.03014 |
| P15056 | T104 | IGDFGLATVKSRWSG | -1.69026 | 0.98866 | 0.01134 | 1.00000 | 0.00262 | 0.65619 | 0.00172 |
| P15056 | S339 | GQRDRSSSAPNVHIN | -1.27608 | 0.91781 | 0.08219 | 0.99850 | 0.34324 | 0.65520 | 0.22523 |
| O14543 | Y208 | VNGHLDSYEKVTQLP | -2.07321 | 0.99980 | 0.00020 | 1.00000 | 0.00000 | 0.65619 | 0.00000 |
| O14543 | Y225 | PGPIREFLDQYDAPL | 1.54126 | 0.04149 | 0.95851 | 0.08069 | 0.99996 | 0.05295 | 0.65616 |
| P01100 | S362 | AAAHRKGSSSNEPSS | -1.46756 | 0.96721 | 0.03279 | 0.99994 | 0.09822 | 0.65615 | 0.06445 |
| P01100 | T232 | GGLPEAATPESEEAF | -1.67349 | 0.99962 | 0.00038 | 1.00000 | 0.00004 | 0.65619 | 0.00003 |

## A.3 Kinome Data for C43.4

| | | | | | | | Negative predictive value down | |
|---|---|---|---|---|---|---|---|---|
| ID | Accession | FC | P up | P down | Beta up | Beta down | | Negative predictive value up |
| P42345 | S1261 | -1.29592 | 0.97134 | 0.02866 | 1.00000 | 0.03183 | 0.64186 | 0.02043 |
| Q8N122 | S863 | -1.63799 | 0.99948 | 0.00052 | 1.00000 | 0.00031 | 0.64187 | 0.00020 |
| Q13131 | S486 | 1.12863 | 0.03060 | 0.96940 | 0.33261 | 0.99863 | 0.21349 | 0.64099 |
| P35568 | S1095 | -1.75880 | 0.99995 | 0.00005 | 1.00000 | 0.00000 | 0.64187 | 0.00000 |
| P35568 | S312 | 1.33513 | 0.00419 | 0.99581 | 0.02092 | 1.00000 | 0.01343 | 0.64186 |
| P48736 | T607 | -1.43568 | 0.93958 | 0.06042 | 0.99971 | 0.19130 | 0.64168 | 0.12279 |
| P48736 | S582 | -1.65658 | 0.99505 | 0.00495 | 1.00000 | 0.01262 | 0.64186 | 0.00810 |
| P42338 | Y505 | 1.19539 | 0.02685 | 0.97315 | 0.23052 | 0.99952 | 0.14796 | 0.64156 |
| P42338 | Y963 | -1.30925 | 0.94124 | 0.05876 | 0.99993 | 0.10729 | 0.64182 | 0.06886 |
| P42338 | S1071 | -1.57462 | 0.97933 | 0.02067 | 0.99992 | 0.11231 | 0.64181 | 0.07209 |
| O00329 | S1021 | -1.79495 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 | 0.00000 |
| P00533 | Y846 | 1.34666 | 0.00395 | 0.99605 | 0.07506 | 0.99997 | 0.04818 | 0.64185 |
| P00533 | Y1172 | -1.83275 | 0.99622 | 0.00378 | 1.00000 | 0.00019 | 0.64187 | 0.00012 |
| Q15831 | S309 | -1.45061 | 0.99660 | 0.00340 | 1.00000 | 0.00752 | 0.64186 | 0.00483 |
| Q15831 | S183 | 1.54843 | 0.00858 | 0.99142 | 0.00869 | 1.00000 | 0.00558 | 0.64186 |
| P60484 | S346 | -1.47335 | 0.98569 | 0.01431 | 1.00000 | 0.00684 | 0.64186 | 0.00439 |
| P60484 | Y104 | -1.59003 | 0.99850 | 0.00150 | 1.00000 | 0.00039 | 0.64187 | 0.00025 |
| O15530 | S242 | -2.10767 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 | 0.00000 |
| O15530 | Y374 | 1.48158 | 0.01912 | 0.98088 | 0.04558 | 0.99999 | 0.02926 | 0.64186 |
| P17252 | Y562 | 1.60337 | 0.00003 | 0.99997 | 0.00006 | 1.00000 | 0.00004 | 0.64187 |
| P17252 | T401 | 1.65075 | 0.00306 | 0.99694 | 0.01102 | 1.00000 | 0.00707 | 0.64186 |
| Q02156 | S368 | 2.00517 | 0.00001 | 0.99999 | 0.00000 | 1.00000 | 0.00000 | 0.64187 |
| Q04759 | T538 | -1.23781 | 0.97086 | 0.02914 | 0.99998 | 0.05481 | 0.64186 | 0.03518 |
| Q04759 | S695 | -1.65632 | 0.98804 | 0.01196 | 1.00000 | 0.00355 | 0.64187 | 0.00228 |
| Q04759 | Y90 | -1.65532 | 0.99897 | 0.00103 | 1.00000 | 0.00017 | 0.64187 | 0.00011 |
| Q9Y243 | S120 | -1.56940 | 0.99969 | 0.00031 | 1.00000 | 0.00130 | 0.64187 | 0.00083 |
| Q9Y243 | S472 | -1.28061 | 0.95348 | 0.04652 | 0.99974 | 0.18358 | 0.64170 | 0.11783 |
| Q9Y243 | T447 | -1.34746 | 0.96580 | 0.03420 | 0.99927 | 0.26801 | 0.64140 | 0.17203 |
| Q9Y243 | T305 | -1.59213 | 0.99110 | 0.00890 | 1.00000 | 0.00222 | 0.64187 | 0.00142 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| P14625 | S347 | -1.86881 | 0.99152 | 0.00848 | 1.00000 | 0.00870 | 0.64186 | 0.00559 |
| P14625 | T165 | -3.20505 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 | 0.00000 |
| P23443 | S394 | -1.92465 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 | 0.00000 |
| P23443 | T412 | 1.27522 | 0.02321 | 0.97679 | 0.09876 | 0.99994 | 0.06339 | 0.64183 |
| Q13541 | S101 | -1.42447 | 0.98688 | 0.01312 | 0.99997 | 0.07800 | 0.64184 | 0.05007 |
| Q13541 | S65 | -1.44205 | 0.99909 | 0.00091 | 1.00000 | 0.00394 | 0.64187 | 0.00253 |
| Q13541 | T46 | -2.03422 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 | 0.00000 |
| Q99558 | T558 | -1.21100 | 0.94038 | 0.05962 | 0.99978 | 0.17237 | 0.64172 | 0.11064 |
| O15111 | S181 | 1.33433 | 0.00063 | 0.99937 | 0.13662 | 0.99987 | 0.08769 | 0.64178 |
| Q14164 | S666 | -1.14878 | 0.95298 | 0.04702 | 0.99943 | 0.24502 | 0.64150 | 0.15727 |
| Q14164 | S172 | -1.63503 | 0.99999 | 0.00001 | 1.00000 | 0.00012 | 0.64187 | 0.00008 |
| P25963 | S283 | 1.89164 | 0.00019 | 0.99981 | 0.00000 | 1.00000 | 0.00000 | 0.64187 |
| P25963 | T291 | -2.76633 | 0.99516 | 0.00484 | 1.00000 | 0.00051 | 0.64187 | 0.00033 |
| P68400 | Y182 | 1.85201 | 0.00100 | 0.99900 | 0.00082 | 1.00000 | 0.00052 | 0.64187 |
| P19838 | S905 | -1.22148 | 0.90069 | 0.09931 | 0.99954 | 0.22604 | 0.64157 | 0.14509 |
| P19838 | S337 | 2.60878 | 0.00000 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 |
| P19838 | S928 | 1.74371 | 0.00000 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 |
| Q00653 | S708 | 1.16606 | 0.01783 | 0.98217 | 0.19025 | 0.99971 | 0.12212 | 0.64168 |
| Q00653 | S99 | -1.22519 | 0.99288 | 0.00712 | 0.99995 | 0.09561 | 0.64183 | 0.06137 |
| P06493 | T161 | 1.43621 | 0.00262 | 0.99738 | 0.00082 | 1.00000 | 0.00053 | 0.64187 |
| P24941 | S46 | 1.39858 | 0.00647 | 0.99353 | 0.00723 | 1.00000 | 0.00464 | 0.64186 |
| P24941 | Y15 | 1.28540 | 0.04064 | 0.95936 | 0.15563 | 0.99983 | 0.09989 | 0.64175 |
| P24941 | T160 | 1.28857 | 0.09519 | 0.90481 | 0.24510 | 0.99943 | 0.15732 | 0.64150 |
| P36897 | T189 | -1.91374 | 0.99989 | 0.00011 | 1.00000 | 0.00001 | 0.64187 | 0.00001 |
| Q15797 | S187 | 1.48976 | 0.00303 | 0.99697 | 0.02657 | 1.00000 | 0.01705 | 0.64186 |
| Q15797 | S532 | 1.22464 | 0.05781 | 0.94219 | 0.15086 | 0.99984 | 0.09683 | 0.64176 |
| Q15797 | S206 | 1.17086 | 0.09275 | 0.90725 | 0.54279 | 0.99298 | 0.34840 | 0.63736 |
| Q15796 | S467 | 1.50434 | 0.01579 | 0.98421 | 0.08191 | 0.99996 | 0.05257 | 0.64184 |
| P84022 | T133 | -1.23550 | 0.92689 | 0.07311 | 0.99922 | 0.27400 | 0.64137 | 0.17587 |
| P17612 | T201 | -1.39747 | 0.99643 | 0.00357 | 1.00000 | 0.01288 | 0.64186 | 0.00827 |
| P17612 | S339 | -1.53132 | 0.99984 | 0.00016 | 1.00000 | 0.00018 | 0.64187 | 0.00011 |
| P10721 | Y569 | -2.00115 | 0.99991 | 0.00009 | 1.00000 | 0.00002 | 0.64187 | 0.00001 |
| P10721 | Y937 | -1.46164 | 0.99994 | 0.00006 | 1.00000 | 0.00023 | 0.64187 | 0.00015 |
| P10721 | Y722 | -1.17023 | 0.95349 | 0.04651 | 0.99937 | 0.25474 | 0.64146 | 0.16351 |
| P29353 | S36 | -1.84108 | 0.96881 | 0.03119 | 0.99995 | 0.09569 | 0.64183 | 0.06142 |
| P29353 | Y427 | -2.42152 | 0.99896 | 0.00104 | 1.00000 | 0.00173 | 0.64187 | 0.00111 |
| Q07889 | S1134 | 1.52478 | 0.00821 | 0.99179 | 0.01449 | 1.00000 | 0.00930 | 0.64186 |
| Q07889 | S1197 | 1.39768 | 0.02461 | 0.97539 | 0.08617 | 0.99996 | 0.05531 | 0.64184 |
| Q07889 | T158 | -1.49110 | 0.99019 | 0.00981 | 1.00000 | 0.00290 | 0.64187 | 0.00186 |
| P01112 | T35 | -1.80295 | 0.99597 | 0.00403 | 1.00000 | 0.00784 | 0.64186 | 0.00503 |
| P01112 | T144 | 1.32245 | 0.00329 | 0.99671 | 0.05520 | 0.99998 | 0.03543 | 0.64185 |
| P01112 | S183 | 1.28485 | 0.00861 | 0.99139 | 0.10525 | 0.99993 | 0.06756 | 0.64182 |
| P46940 | S282 | 1.16688 | 0.07235 | 0.92765 | 0.27250 | 0.99924 | 0.17491 | 0.64137 |
| P46940 | T1392 | -1.74884 | 0.99999 | 0.00001 | 1.00000 | 0.00000 | 0.64187 | 0.00000 |
| P46940 | Y1459 | -2.89224 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 | 0.00000 |
| P63000 | T96 | 2.27267 | 0.00000 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 |
| P04049 | S338 | 1.62717 | 0.00207 | 0.99793 | 0.10856 | 0.99993 | 0.06968 | 0.64182 |
| P04049 | S43 | -1.20012 | 0.95459 | 0.04541 | 0.99985 | 0.14585 | 0.64177 | 0.09362 |
| P04049 | S259 | -1.32716 | 0.99691 | 0.00309 | 1.00000 | 0.01211 | 0.64186 | 0.00777 |
| P17181 | S536 | -2.04312 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 | 0.00000 |
| P17181 | Y482 | 1.42979 | 0.00043 | 0.99957 | 0.00595 | 1.00000 | 0.00382 | 0.64186 |
| P17181 | Y467 | 1.23497 | 0.01793 | 0.98207 | 0.10514 | 0.99993 | 0.06749 | 0.64182 |
| P23458 | T686 | 2.15356 | 0.00000 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| P23458 | Y1033 | 4.44389 | 0.00000 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 |
| P42224 | Y1114 | -2.28319 | 0.99999 | 0.00001 | 1.00000 | 0.00000 | 0.64187 | 0.00000 |
| P40763 | S727 | 1.30038 | 0.01276 | 0.98724 | 0.04129 | 0.99999 | 0.02650 | 0.64186 |
| Q13153 | S198 | -1.92179 | 0.99634 | 0.00366 | 1.00000 | 0.00272 | 0.64187 | 0.00174 |
| Q13153 | Y284 | 1.54170 | 0.00009 | 0.99991 | 0.00446 | 1.00000 | 0.00286 | 0.64187 |
| Q13177 | S141 | -1.09723 | 0.93999 | 0.06001 | 0.99893 | 0.30599 | 0.64118 | 0.19640 |
| O96013 | S475 | -1.41050 | 0.99272 | 0.00728 | 0.99998 | 0.05527 | 0.64185 | 0.03548 |
| O96013 | S181 | 1.31202 | 0.00126 | 0.99874 | 0.01841 | 1.00000 | 0.01182 | 0.64186 |
| O96013 | S99 | 1.25986 | 0.03162 | 0.96838 | 0.23029 | 0.99952 | 0.14781 | 0.64156 |
| Q02750 | S222 | 1.92785 | 0.00007 | 0.99993 | 0.00003 | 1.00000 | 0.00002 | 0.64187 |
| P36507 | S306 | 1.38031 | 0.00190 | 0.99810 | 0.02270 | 1.00000 | 0.01457 | 0.64186 |
| P36507 | S226 | 1.29689 | 0.00806 | 0.99194 | 0.01679 | 1.00000 | 0.01078 | 0.64186 |
| P36507 | T398 | -1.32680 | 0.99289 | 0.00711 | 1.00000 | 0.01509 | 0.64186 | 0.00969 |
| P28482 | S246 | -1.76240 | 0.99537 | 0.00463 | 1.00000 | 0.00173 | 0.64187 | 0.00111 |
| P28482 | Y187 | -1.25596 | 0.90398 | 0.09602 | 0.99924 | 0.27178 | 0.64138 | 0.17445 |
| P51812 | S386 | 1.93158 | 0.00087 | 0.99913 | 0.00031 | 1.00000 | 0.00020 | 0.64187 |
| Q15418 | T359 | 1.48848 | 0.00043 | 0.99957 | 0.00034 | 1.00000 | 0.00022 | 0.64187 |
| P05412 | Y170 | -1.16658 | 0.96480 | 0.03520 | 0.99757 | 0.40039 | 0.64030 | 0.25700 |
| Q16539 | Y323 | -1.52622 | 0.99346 | 0.00654 | 1.00000 | 0.00102 | 0.64187 | 0.00066 |
| Q16539 | Y182 | -1.32150 | 0.95991 | 0.04009 | 0.99981 | 0.16003 | 0.64175 | 0.10272 |
| P49137 | T322 | 1.69502 | 0.00001 | 0.99999 | 0.00000 | 1.00000 | 0.00000 | 0.64187 |
| P49137 | S259 | 2.45711 | 0.00005 | 0.99995 | 0.00000 | 1.00000 | 0.00000 | 0.64187 |
| P49137 | T209 | 1.38802 | 0.07592 | 0.92408 | 0.24664 | 0.99942 | 0.15831 | 0.64149 |
| P42574 | S150 | -2.36038 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 | 0.00000 |
| P63104 | S58 | 1.26221 | 0.00052 | 0.99948 | 0.00320 | 1.00000 | 0.00206 | 0.64187 |
| P63104 | T110 | 1.90184 | 0.00397 | 0.99603 | 0.00350 | 1.00000 | 0.00225 | 0.64187 |
| P11274 | S120 | -1.59722 | 0.99936 | 0.00064 | 1.00000 | 0.00023 | 0.64187 | 0.00015 |
| P11274 | Y21 | 4.30017 | 0.00000 | 1.00000 | 0.00000 | 1.00000 | 0.00000 | 0.64187 |

### A.4 Phenol Chloroform DNA Purification from *E. coli*

1. Grow cultures in LBA overnight at 37$^{o}$C with agitation
2. Centrifuge media at 10,000 rpm, discard supernatant
3. Resuspend pellet in glucose-Tris-EDTA buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) and incubate for 5 min at RT
4. Add lysis buffer to a final concentration of 0.13 M NaOH, 0.66% SDS then incubate for 5 min on ice
5. Add NaAc pH 4.8 to a final concentration of 1 M and incubate for 5 min on ice
6. Centrifuge at 18,000 rpm and 4$^{o}$C for 5 min
7. Decant supernatant and add 1:2 v/v isopropanol:supernatant
8. Incubate on ice for 5 min then centrifuge at 18,000 rpm and 4$^{o}$C for 5 min
9. Discard supernatant and resuspend pellet in 2:1 v/v 6.6 M LiCl:phenol-chloroform
10. Vortex and incubate at RT for 5 min
11. Centrifuge at 14,800 rpm for 2 min and extract aqueous layer
12. Add 3X 95% Ethanol (EtOH) and incubate at -80$^{o}$C for 30 min
13. Centrifuge at 18,000 rpm and 4$^{o}$C for 5 min then discard supernatant
14. Add 70% ice cold EtOH and centrifuge 18,000 rpm and 4$^{o}$C for 5 min
15. Discard supernatant then resuspend pellet in 1X TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0)

## A.5 Gel Extraction Purification

1. Run endonuclease reaction on a 1.5% low melt agarose gel
2. Excised fragments from the gel and combine with 2.5X volume 50 mM NaCl
3. Incubate at 65$^{o}$C for up to 10 min with intermittent vortexing
4. Mix gel/NaCl solution 1:1 v/v with phenol
5. Vortex then centrifuged at 14,800 rpm for 2 min.
6. Decant supernatant
7. Repeat steps 4-6 until no interface remains.
8. Mix supernatant 1:1 v/v with n-butanol, vortex, and centrifuge at 14,800 rpm for 2 min
9. Extract the aqueous layer and mix with 3X 95% EtOH then incubate at -80$^{o}$C for 30 min
10. Centrifuge at 18, 000 rpm and 4$^{o}$C for 10 min.
11. Decant supernatant and resuspend DNA in 1X TE